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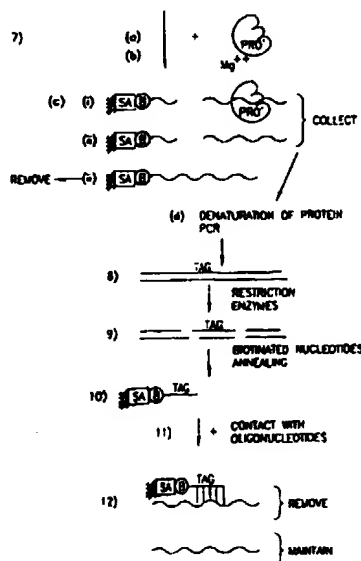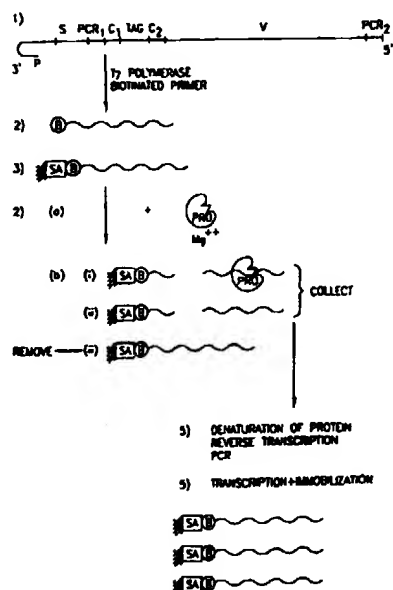
(57) Abstract

The present invention concerns nucleic acid molecules, or complex of molecules, which have no catalytic activity in the absence of a specific co-factor, and feature catalytic activity only in the presence of a specific co-factor. The present invention further concerns methods

CATALYTIC NUCLEIC ACID AND ITS MEDICAL USE

FIELD OF THE INVENTION

The present invention concerns molecules and methods for detection of specific agents in a medium.

5 ## BACKGROUND OF THE INVENTION

Ribozymes are typically RNA molecules which have enzyme–like catalytic activities usually asociated with cleavage, splicing or ligation of nucleic acid sequences. The typical substrates for ribozymes catalytic activities are RNA molecules, although ribozymes may catalyze reaction in

10 which DNA molecules (or maybe even proteins) serve as substrates.

Ribozymes which are active intracellularly work in *cis*, catalyzing only a single turnover, and are usually self–modified during the reaction. However, ribozymes can be engineered to act in *trans*, in a truly catalytic manner, with a turnover greater than one and without being self–modified.

15 Two distinct regions can be identified in a ribozyme: the binding region which gives the ribozyme its specificity through hybridization to a specific nucleic acid sequence (and possibly also to specific proteins), and a catalytic region which gives the ribozyme the activity of cleavage, ligation or splicing.

20 It has recently been proposed to use ribozymes in order to treat diseases or genetic disorders by cleaving a target RNA, such as viral RNA or messenger RNA transcribed from genes that should be turned off. This was proposed as an alternative to blockage of the RNA transcript by the use of antisense sequences. Owing to the catalytic nature of the ribozyme, a

25 single ribozyme molecule cleaves many molecules of target RNA and therefore therapeutical activity is achieved in relatively lower concentrations

The use of ribozymes for diagnostic purposes has been only seldomly mentioned. WO 94/13833 describes a method for detecting nucleic acid molecules in a solution by tailoring a specific ribozyme molecule having two regions, one complementary to the nucleic acid sequence to be detected, and the other complementary to a co-target molecule bearing a detectable label. The ribozyme is able to specifically and reversibly bind both to a selected target nucleic acid sequence and to the labelled co-target. When both the target and the co-target are bound, the ribozyme undergoes a conformational change which renders it active and able to cleave the label of the co-target, and the free label can then be detected. Upon cleavage of the co-target, the ribozyme is able to re-associate with an additional co-target, cleaving more label and producing more detectable signals.

WO 94/13791 concerns a regulatable ribozyme molecule which upon binding to a ligand alters its activity on a target RNA sequence. Again, as in WO 94/13833, binding to the target causes a conformational change in the ribozymes which renders it active. An example for such a change, is in the presence in the ribozyme of a redundant sequence which masks the core region of the ribozyme. Only upon binding of the target sequence to said redundant sequence, the core becomes unmasked, and thus active.

U.S. Patent 5,472,840 (Stefano, J.E.) discloses a nucleic acid sequence comprising the MDV-1 motif (capable of autocatalytic replication in the presence of the enzyme of the Q-beta replicase) which becomes active only when it forms a specific structure, comprising the sequence GAAA, with a target nucleic acid molecule. The ribozyme of Stefano is prepared by modifying an existing ribozyme and features only the activity of cleavage in the presence of a very restricted range of target molecules.

Lizardi, M.P. and Helena Porta (*Biotechnology* 13:161-164 (1995)) disclose an allosteric hammerhead ribozyme that is *a priori* inactive, and is triggered by specific interaction with a DNA allosteric effector that

This publication discloses a ribozyme which becomes active when a part of which becomes double-stranded.

The above publications, disclose ribozymes which become catalytically active, either due to a conformational change, or due to a hybridization reaction which render them double-stranded. These types of reactions, may also occur spontaneously, for example, if a ribozyme is inactive due to the presence of a redundant sequence which masks its core region, this redundant sequence may either break, or open, even in the absence of the target, and thus the ribozyme will become catalytically active even without a target. Spontaneous reversion to an active state, of course render the ribozymes impractical for diagnostic purposes.

Israel Patent Applications 112799 and 115772 (corresponding to PCT/US96/02380) disclose methods for the detection of catalytically active ribozymes in a medium, wherein typically the catalytically active ribozyme is used as a reporter for the presence of other biomolecules in a test sample. In accordance with the methods disclosed in these applications, catalytically active ribozyme, if present in an assayed medium, yields a reaction cascade in which more catalytically active ribozymes are produced in a positive-feedback manner in one of various embodiments specified in these applications. The catalytically active ribozyme may be produced, or activated only in the presence of an assayed molecule.

GENERAL DESCRIPTION OF THE INVENTION

In the following, the term *"nucleozyme"* will be used to denote an oligonucleotide or a complex formed between an oligonucleotide and a nucleic acid sequence or between an oligonucleotide and another molecule e.g. an oligonucleotide, a protein or a polypeptide, etc., which possesses a catalytic activity. An example of a nucleozyme is a ribozyme.

The term *"proto-nucleozyme"* will be used to denote a nucleic acid molecule or a complex of two or more such molecules, which ☐has *a priori* no catalytic activity but which becomes catalytically active upon

a nucleozyme with a missing component, which missing component is completed by the co-factor. The complex between the proto-nucleozyme and the co-factor may also at times be referred to as "catalytic complex" and is in fact a nucleozyme since it has catalytic activity. The proto-nucleozyme may consist of deoxynucleotides (dNTP's), ribo-nucleotides (rNTP's), as well as other nucleotides such as 2'-O-methyl nucleotides, or any combinations of these.

The term "catalytic activity" is meant to encompass all possible catalytic activities of nucleozymes, including cleavage, ligation, splicing-out (cleaving both ends of a short nucleic acid sequence to remove it from a longer sequence and ligating the ends of the cut), splicing-in (cleaving open a nucleic acid sequence, inserting another short nucleic acid sequence and ligating the ends of the cut), rearrangement, as well as additional catalytic activities such as phosphorylation, kinase like activity, addition or removal of other chemical moieties, biotinilation, gap filling of missing nucleotides, polymerization, etc.

The term "co-factor" will be used to denote a molecule or a moiety within a molecule (e.g. a certain DNA sequence within a larger DNA molecule), which complexes with the proto-nucleozyme to yield a catalytic complex, namely an active nucleozyme. The co-factor completes a missing portion of the proto-nucleozyme so that it can become catalytically active, and turn into a nucleozyme.

The present invention relates to novel proto-nucleozymes and their use. The proto-nucleozymes of the invention have substantially no catalytic activity, as they miss a critical component, which is essential for the catalytic activity, said missing component is completed by the co-factor. In other words, in order to become catalytically active, the proto-nucleozymes need to complex with the co-factor, so as to form a catalytic complex (the nucleozyme) which can exert a catalytic activity. The term "having substantially no catalytic activity" is meant to denote that the proto-nucleozyme possesses either no catalytic activity or possesses a catalytic

activity which is very much lower (typically by several orders of magnitude) than that of the catalytic complex.

As pointed out above, there are known ribozymes which complex with a factor (termed also target), e.g. a protein or another nucleic acid sequence which causes the ribozymes to undergo conformational change whereby the catalytic activity of the ribozyme becomes more pronounced. However, in distinction from such prior art ribozymes, the proto-nucleozyme of the invention, *a priori* misses an essential component and said co-factor provides said missing component. Examples of such missing components are sequences in the core region of the nucleozyme. The co-factor may be a protein which fills a gap between two ends of nucleic acid strands of the nucleozyme or which joins such two ends; or may be a nucleic acid sequence capable of binding between two free ends of the proto-nucleozyme thus filling a gap.

Given the structural and functional features of the proto-nucleozyme, when in solution, it does not have the capability of spontaneously converting into a catalytically active form, since its activation is not dependent solely on a conformational change which may occur spontaneously but rather on completion of a missing component. This feature is a further distinction from prior art inactive ribozymes, which have the capability to spontaneously undergoing conformational change, albeit at a low rate, and exert some catalytic activity even in the absence of a co-factor. Thus, unlike prior art inactive ribozymes, such as those described above in the Background Of The Invention section of this specification, when the ribozymes of the invention are used, there is substantially no background activity in the absence of the co-factor. For example, when proto-nucleozymes of the invention are used in diagnosis, there is essentially no *"noise"*, namely, there is a very high signal-to-noise ratio and there are virtually no false-positive results. By another example, when used in therapeutics, the proto-nucleozymes of the invention, will exert a very high target specificity and only in the presence of an appropriate target, the

The proto–nucleozymes of the invention can be prepared both by *in vitro* evolution, in a manner to be described below, or by means of rational design by nucleic acid synthesis, for example, by using a sequence of a known ribozyme and leaving a gap of several missing nucleotides in its

5   core region.

The present invention provides a proto–nucleozyme, being a nucleic acid molecule or a complex of nucleic acid molecules, having essentially no catalytic activity but can complex with a co–factor to form a nucleozyme which possesses a catalytic activity, the proto–nucleozyme lacks

10   a component essential for the catalytic activity of the nucleozyme and said co–factor provides said component.

The component which is missing in the proto–nucleozyme and which is provided by the co–factor for conversion of the proto–nucleozyme into a catalytically active nucleozyme may be a missing nucleic acid

15   segment of one or more nucleotides (hereinafter *"gap"*) or a missing bond between two nucleotides (hereinafter *"nick"*), etc. The co–factor may thus be a nucleic acid segment which can bridge the gap or nick. For this purpose, such a co–factor nucleic acid segment has sequences which can hybridize with complementary sequences in the two strands on both sides of

20   the gap or nick and in the case of a gap, may comprise also the missing sequence. It should be noted that at times the co–factor segment may hybridize to and provide a bridge between two nucleic acid stretches of the proto–nucleozyme other than those immediately flanking the gap or nick. In addition, the co–factor may also at times be a macromolecule such as a

25   protein, an oligosaccharide, etc., which can complex with the two nucleic acid terminals flanking the gap or nick and accordingly bring to bridging of the two. The gap or nick may be between two different oligonucleotide strands which are functionally joined together by said co–factor or may be between ends of the same oligonucleotide wherein the co–factor in such

30   yields the function of a functional closed oligonucleotide loop.

Such proto–nucleozymes may be obtained (*"engineered"*) by

The specific co-factor may be any molecule which can chemical-
ly interact with nucleotides in any manner, e.g. by the formation of
hydrogen bond, by electrostatic bonds, by Van der Waals' interactions, etc.
Such molecules include, proteins, peptides, oligopeptides, antibiotics,
5   phosphate nucleotides such as ATP, GTP, cyclic AMP, and others,
carbohydrates, lipids, nucleic acid sequences (DNA or RNA sequences), etc.

At times it may be desired to modify the proto-nucleozyme in
order to increase its nuclease resistance, which may be achieved by replacing
some of the natural nucleotides, by non-natural nucleotides such as 2'-O-
10   methyl nucleotides (Usman *et al., Nucleic Acids Symposium Series*, 31:163-
164, 1994).

A currently preferred, but not exclusive, use of the proto-
nucleozymes of the invention is as a diagnostic tool for the detection of the
presence of a certain agent in a medium. For this purpose, a proto-
15   nucleozyme will be designed such that said agent will be the specific co-
factor. When said agent will be present in the tested medium, a catalytic
complex will form and the catalytic activity, which will then be assayed,
will then serve as a gauge for the presence of said agent in the medium.

For this preferred embodiment, it is preferred to use proto-
20   nucleozymes which possess absolutely no or unmeasurable catalytic activity,
and the catalytic activity is essentially manifested then only upon the
formation of the catalytic complex. In such case, there will be an essentially
zero background signal and a detection of a catalytic activity will then be an
unequivocable (*"all or none"*) indication of the presence of said agent (which
25   is the specific co-factor) in the medium. Where a proto-nucleozyme is
used which possesses some small catalytic activity, the level of the catalytic
activity will have to be determined in order to assay the presence of said
agent in the medium.

The present invention further provides a method for the detection
30   of an agent in a medium, which comprises the following steps:

(a)   contacting said medium with a proto–nucleozyme, wherein said agent
       is the specific co–factor required for the formation of a catalytic
       complex;

(b)   providing or maintaining conditions allowing catalytic activity of the
5   catalytic complex; and

(c)   assaying for the presence of products of the catalytic activity in the
       medium, such presence indicating the presence of said agent in the
       medium.

An example of the type of catalytic activity which may be
10   determined in step (c) above, is cleavage of nucleic acid sequence. For
example, the catalytic complex may cleave, from an immobilized nucleic
acid substrate, a small fragment bearing a detectable label. Then, detection
of a free label in the reaction medium is indicative of the activity of the
catalytic complex, which is in turn an indication of the presence of the
15   assayed agent in the medium.

Other examples of catalytic activity which can be determined in
step (c) are ligation, splicing out, splicing in, etc. All the above three
catalytic activities result in a change in the distance between two sequences
of nucleotides which are the substrates for the reaction. In ligation and
20   splicing out two sequences are brought together and in splicing two
sequences are spaced apart. One of these sequences may bear, for example,
a fluorescent containing moiety (e.g. rhodamine), and the other sequence
may bear a fluorescent quenching (e.g. fluorescein) containing moiety or a
fluorescent enhancing moiety; the change in the distance between the two
25   sequences may then be determined by measuring the change in fluorescence
emission, which is quenched (in the case of a fluorescent quencher) or
enhanced (in the case of a fluorescent enhancer) when the two sequences are
adjacent one another, and enhanced or quenched, respectively, when the two
sequences are spaced apart.

30   One preferred method for assaying for the products of the
catalytic activity is by means of the self amplifying ribozyme cascade

corresponding Israel Patent Applications 112799 and 115772, the contents of which are being incorporated herein by reference. Briefly, the catalytic complex (or the nucleozyme) once formed catalyzes a reaction which brings to activation of *a priori* inactive nucleozymes (identified in the above-

5   referenced applications as ribozymes), and those catalytically active nucleozymes then act catalytically to activate additional ribozymes and so forth, in a self amplifying positive feedback reaction cascade. This amplified signal thus serves as a gauge for the presence of the initial catalytically active nucleozyme in the medium; the presence of such an

10   initial catalytically active nucleozyme is in turn an indication of the presence of the specific co-factor which is the agent to be detected and which complexes with the proto-nucleozymes to yield the catalytic complex of the initial ribozyme in the medium.

The unique capability of the proto-nucleozymes to become

15   catalytically active in the presence of a specific co-factor, makes them useful also as therapeutic agents in certain targeted therapies. In some diseases, disease-bearing cells differ from normal cells in expression of certain expression products. This is the case, for example, in viral diseases where diseased cells differ from other cells by the fact that they express

20   viral proteins. The proto-nucleozyme may be engineered so that upon complexation with a viral-specific protein, (which will be the specific co-factor), it will have a certain cytotoxic catalytic activity or its catalytic activity will yield a cytotoxic reaction product. The catalytic activity may be targeted at a specific nucleic acid sequence or a gene expression product

25   such that the undesired gene expression (e.g. of a viral origin or of a cellular origin) will be inhibited. For example, the catalytic activity may cleave the sequence of an undesired mRNA, thus inhibiting production of an undesired protein. Such proto-nucleozymes may be provided in formulations allowing their entry into the cell, e.g. a liposome formulation, and while the proto-

30   nucleozymes will enter many cells, they will assume their catalytic activity and will thus destroy only the desired cell population for example, or inhibit

In addition to viral diseases, there are also other diseases where diseased cell express or contain products which are not found in normal cells or found in the latter cells in only small amounts. Such is the case, for example, in cancer; in a variety of infectious diseases other than viral

5   diseases; in various genetic diseases where diseased cells in diseased individuals contain a mutant gene and abnormal expression products; etc.

Accordingly, the present invention provides a method for selectively destroying a specific cell population, containing or expressing a specific agent, the method comprising:

10   (a)   providing a proto-nucleozyme of a nucleozyme, which nucleozyme has a catalytic activity which is cytotoxic to the cell or has cytotoxic reaction products, and wherein said agent is a specific co-factor to said proto-nucleozyme;

       (b)   inserting said proto-nucleozyme to cells containing or expressing said

15   agent, or applying said proto-nucleozymes to tissue, suspected of containing a population of cells containing or expressing said agent, under conditions or using a vehicle, so as to insert said proto-nucleozyme to said cells.

In the case where the proto-nucleozyme is intended for destroy-

20   ing viral-containing cells, the co-factor may, for example, be a viral-associated protein, e.g. in the case of HIV, the HIV-TAT protein.

The cytotoxicity of the nucleozyme may be manifested in a variety of ways. For example, a nucleozyme, once becoming active, may catalyze a reaction yielding formation of cytotoxic reaction products. Such

25   cytotoxic reaction products may, for example, be reaction production which competitively inhibit one or more essential metabolic or catabolic pathways in the cells. By another example, the catalytic activity of the cytotoxic ribozyme may by itself yield breakdown of substances which are produced within the cells or transported into the cell through the cells' membranes,

30   which are essential for the cells' growth and survival. Other examples may be nucleozymes which degrade mRNA, either in general or such having

specific sequences, nucleozymes which breakdown tRNAs, nucleozymes which degrade a variety of proteins, and others.

The present invention also provides a method for inhibiting expression of undesired genes in cells, comprising:

5   (a)   providing a proto-nucleozyme of a nucleozyme, which nucleozyme has a catalytic activity which is targeted at a specific nucleic acid sequence or gene expression product so that once active within a cell, the undesired gene expression will be inhibited;

     (b)   inserting said proto-nucleozyme to cells containing or expressing said
10   gene, or applying said proto-nucleozyme to tissues suspected of having a population of cells containing or expressing said gene, under conditions or using a vehicle so as to insert said proto-nucleozyme to said cells.

By an embodiment of this latter aspect, the co-factor of the
15   proto-nucleozyme is either a nucleic acid sequence of such gene, or a transcription or expression product of the gene.

The inhibition of the DNA expression by this latter method may, for example, by breakdown of the specific mRNA, breakdown of an expression product of the gene, or breaking down regulatory substances
20   regulating expression of the gene.

The above methods where said proto-nucleozyme is inserted into cells, may be useful in human therapy. The proto-nucleozymes within the framework of such therapies may be administered *in vivo* or may be contacted with cells *ex vivo*, which cells are then inserted back into the
25   body.

According to its therapeutic aspect, the present invention further provides a pharmaceutical composition e.g. for use in destroying a specific cell population, which comprises said proto-nucleozyme and a pharmaceutically acceptable carrier. The carrier will preferably be of a kind which
30   allows insertion of the proto-nucleozyme into cells, e.g. a liposome carrier or any other carrier known in the art.

As explained above, the nucleozyme of the invention may be produced by rational design or by way of *in vitro* evolution.

As will be explained further below, *in vitro* evolution does not require any decision in advance on the exact mechanism of formation of the catalytic complex between the proto-nucleozyme and the specific co-factor. The exact mechanism of activation evolves during such *in vitro* evolution.

The term *"in vitro evolution"*, refers to a method of generating and selecting nucleic acid sequences (which may be DNA or RNA sequences, or sequences comprising both dNTP's and rNTP's comprising naturally or non-naturally occurring nucleotides) having desired characteristics, without *a priori* knowing the exact construct of the selected nucleic acid sequence. Typically, it entails production of a huge number of random, or partially random, nucleic acid sequences or complexes comprising one or more nucleic acid sequences, then providing the conditions required for selection of those sequences which feature a specific property (for example, adding a protein and selecting only those nucleic acid sequences which show a catalytic activity only in the presence of the protein). The selected nucleic acid sequences are then amplified, for example, by polymerase chain reaction (PCR), and then selection and amplification steps are repeated over many cycles, e.g. ranging from 10 to 100, resulting in an enrichment of the reaction mixture by those nucleic acid sequences or complexes which feature the desired property. It is at times useful to progressively increase the threshold criteria for selection in each round of a selection and amplification. For example, as the steps of the *in vitro* evolution proceeds, only species having progressively higher affinity to the desired protein are selected.

In vitro selection methodologies to probe RNA function and structures are summarized in a review by Conrad, R.C., *Molecular Diversity* 1:69-78 (1995) and have been studied in models for autocatalytic replication of RNA by Giver *et al.* (G.R. Fleischaker *et al.* (Editor), *Self-Production of Supramolecular Structures* 137-146, (1994), Klewer Academic Publishers).

The present invention thus provides an *in vitro* evolution method for the production of a proto-nucleozyme of the invention which upon formation of a catalytic complex with a specific co-factor manifests a specific catalytic activity which is either absent or considerably lower in the proto-nucleozyme. The method which has both positive and negative selection steps, comprises:

(a) preparing a panel of different nucleic acid molecules, at least part of the sequence in the molecules of the panel is a random or a partially random sequence, such that said part has a different sequence in different molecules of the panel;

(b) adding said co-factor to said panel and incubating under conditions permitting said catalytic activity;

(c) separating between the nucleic acid molecule of said panel which feature said catalytic activity and such which do not, to obtain a first selected panel of nucleic acid molecules which feature said catalytic activity;

(d) amplifying the nucleic acid molecules of said first selected panel;

(e) incubating said first selected panel with a reaction mixture, being devoid of said co-factor, under conditions permitting said catalytic activity;

(f) removing nucleic acid molecules which featured catalytic activity, thereby obtaining a second selected panel of nucleic acid molecules devoid of the removed nucleic acid molecules; and

(g) repeating steps (b)-(f) over a plurality of cycles, e.g. about 10-100 cycles, to obtain said proto-nucleozyme.

By one embodiment, the molecules in the panel are comprised of an entirely random sequence with the exception of two short flanking sequences for attachment of the PCR primers. By another embodiment of the invention the molecules in the panel are constructed based on a known nucleozyme sequence. For example, the molecule may be comprised of a constant, ribozyme-derived sequence attached to a random or semi-random

sequence. Generally, a random sequence may be prepared, for example, by utilizing a nucleic acid synthesizer.

In the above method, positive selection steps (steps (b)–(d)) precede the negative selection steps (steps (e)–(f)), this being a preferred embodiment of the invention. However, it is possible also to perform the method in a reverse sequence, namely, first the negative selection steps and then the positive selection steps.

Another example is a panel of molecules, all based, on a known ribozyme sequence, and wherein the nucleotides in the entire molecule or in a part thereof have a certain probability (e.g. 70–99%) of being identical to the known sequence. Such a semi–random sequence means that each nucleotide has a certain probability of being different than the corresponding nucleotide in the original ribozyme (e.g. this probability is 30–1%, respectively). The preparation of the panel begins with a known nucleozyme sequence and then semi–random sequences are created based thereon by replacing some of the nucleotides with the different, random, nucleotides ("doping"). The level of doping is typically about 1–30%. The doping may also be performed in each cycle or once in several cycles, thereby slowly bringing to the evolution of a proto–nucleozyme with a high specificity towards the specific co–factor.

An example of how such selection is made, can be drawn from the specific case where the catalytic activity is cleavaged in cis. Initially, all members of the panel may be immobilized on a solid support. Those sequences which possess the catalytic activity of cis cleavage in the positive selection steps i.e. in the presence of the specific co–factor (steps (b)–(d)) are freed to the reaction mixture and are collected and amplified, for example using PCR. After amplification, the collected sequences are allowed to cleave in the absence of the specific co–factor, and those freed in the negative selection steps (steps (e)–(f)), are removed.

In the steps of selection, it is at times desired to apply the exact conditions in which the proto–nucleozyme will eventually be used. Where

diagnostic assay, the conditions of selection will typically be similar to those in the diagnostic assay. For example, where the co-factor is a blood protein, and the proto-nucleozyme is intended for use in a diagnostic assay for detection of such a protein in the blood, the selection (both positive and

5   negative) may be carried out in a reaction medium mimicking the conditions (both the chemical environment and the temperature) which will exist in the assay, e.g. blood-like composition and room temperature.

In the following, a preferred *in vitro* evolution method, useful for the preparation of proto-nucleozymes of the invention, will be described.

10   It should however be noted that this *in vitro* evolution method is an example, and the invention is not limited to the preparation of proto-nucleozymes to this specifically described method, neither to *in vitro* evolution preparation methods in general.

15   **A preferred *in vitro* evolution method**

A major problem in an *in vitro* evolution method for the preparation of a proto-nucleozyme having the above defined features, is the difficulty in separating between candidates of proto-nucleozymes which feature catalytic activity only in the presence of the specific co-factor, and

20   those which feature the specific catalytic activity both in the presence of said co-factor as well as in its absence, or in the presence of other agents. In other words, there is difficulty in eliminating the undesired candidates.

Many times, especially in the initial cycles of the *in vitro* evolution, there are molecules which feature catalytic activity also in the

25   absence of the specific co-factor (which molecules should be removed), but do so at a very low efficiency; thus the negative selection step in which such molecules are removed, may require extremely prolonged incubation times, rendering impractical the whole process of *in vitro* evolution (which requires multiple cycles of negative selection steps). Under standard incubation times

30   only a small percentage of the molecules which are slow acting but feature a catalytic activity also in the absence of the co-factor, but which should be

activity in the given incubation time. This can result in incomplete removal of undesired molecules in the negative selection step, and may eventually lead to false positive results when the proto-nucleozymes are used for diagnosis. In principle, the molecules removed during the negative selection

5   step may be collected, and may be used to *"fish out"*, by hybridization, other identical molecules of the same species of proto-nucleozymes – candidates which should have been removed, but due to the short incubation time did not have a chance to feature the catalytic activity. However, the sequences of candidate proto-nucleozymes which feature catalytic activity both in the

10   presence of the specific co-factor and in the presence of other agents, (to be removed), and the sequences of the candidate proto-nucleozymes which feature the desired property only in the presence of said co-factor (to be maintained), may be very similar (the difference in the sequence of the two may be very small relative to the large size of the candidate proto-

15   nucleozyme) so that it would be practically impossible to distinguish between the two by hybridization.

The solution to this problem is the addition of a random tag sequence to each of the candidate proto-nucleozyme in the initial oligonucleotide mixture comprising a panel of different candidate proto-

20   nucleozymes, so that after amplification, all oligonucleotides of the same species (i.e. all molecules amplified from an original parent molecule) have the same random tag sequence. This tag sequence, does not form a part of the functional sequence of the nucleozyme (i.e. the tag is a redundant sequence). The functional sequence is the part that when the proto-

25   nucleozyme reacts with the co-factor which is the part which imparts the catalytic activity. The tag sequence is linked to a variable sequence (candidate for evolving to the functional sequence). While the variable sequence of different oligonucleotide species may be similar (for example since all variable sequences were *"doped"* from an original known sequence

30   of a ribozyme); the tag sequence is completely different from one oligonucleotide species to the other.

Thus, when, following a negative selection step, oligonucleotides featuring catalytic activity in the absence of said co-factor are collected in order to be removed, it is then possible to first cleave the cleavable sequence, and thus to collect separately only the tag sequences of the

5   undesired oligonucleotides. These tag sequences, which are random, have a very high probability of being different in each species of oligonucleotide molecules, and thus may be used to effectively *"fish out"* complementary tag sequences of other members of the undesired oligonucleotide species to be removed and are thus capable of discovering *"latent"* oligonucleotides which,

10 if the incubation time was long enough, would have featured catalytic activity even in the absence of the co-factor. Since the *"fishing out"* i.e. elimination process is based only on the hybridization of the unique tag sequence (which is different than the tag sequences of the other species), the fact that the variable sequence of the oligonucleotides which are to be

15 removed is very similar to the variable sequence of the oligonucleotides which should be maintained, does not interfere with the selection process.

Thus, the *in vitro* evolution method for obtaining proto-nucleozyme of the invention, which together with a co-factor form a catalytic complex having a catalytic activity imparted by a functional

20 sequence of said proto-nucleozyme, comprises:

(a)   preparing a mixture of different oligonucleotide candidates for evolving to said proto-nucleozymes each of which comprises a variable sequence being a candidate for evolving into said functional sequence and a tag sequence, each of the two sequences being different than corresponding

25 sequences of different oligonucleotides in the mixture, the variable sequence and the tag sequence being linked by at least one cleavable sequence;

(b)   processing the mixture through positive and negative selection steps, each step optionally followed by an oligonucleotide amplification step, there being at least one positive selection step and at least one negative

30 selection step, these steps comprising:

(ba)   a positive selection step comprising applying said specific

and those which do not have catalytic activity to obtain a first selected mixture comprising a first group of oligonucleotides featuring catalytic activity in the presence of the co-factor;

(bb) amplifying said first group of oligonucleotides in said first selected mixture to produce a plurality of copies of each of said first group of oligonucleotides to obtain a first amplified mixture;

(bc) a negative selection step comprising:

(bca) applying a reaction mixture devoid of the specific co-factor and separating between a second group of oligonucleotides which do not have catalytic activity in the absence of the co-factor and a third group of oligonucleotides having the catalytic activity in the absence of the co-factor, to obtain a second selected mixture comprising said second group of oligonucleotides and a third selected mixture comprising said third group of oligonucleotides;

(bcb) amplifying said third group of oligonucleotides in said third selected mixture to produce a plurality of copies of each of said third group of oligonucleotides to obtain a second amplified mixture;

(bcc) cleaving the cleavable sequence of the oligonucleotides in the second amplified mixture, separating between the variable and the tag sequences and collecting the tag sequences;

(bcd) contacting the collected tag sequences with the second selected mixture under stringent conditions of hybridization and removing hybridized oligonucleotides from other oligonucleotides of the second mixture, thereby obtaining a fourth selected mixture of oligonucleotides essentially devoid of oligonucleotides which have catalytic activity in the absence of the co-factor;

where said positive selection step precedes said negative selection step, said positive selection step is applied on said mixture prepared in step (a) and said negative selection step is applied on said first amplified mixture; and where said negative selection step precedes said positive selection step, said negative selection step is applied on said mixture obtained in step (a) and said positive selection step is applied on said fourth mixture.

Preferably, the positive selection step should precede the negative selection step. It is preferable that in the positive selection step of each consecutive cycles the conditions become more and more stringent, for example, shorter assay times, harsher sample preparation conditions etc.; while in the negative selection step conditions become less and less stringent, allowing even those oligonucleotides with a very small catalytic activity in the absence of the co-factor, to exert their catalytic activity (and thus to be removed) for example by utilizing longer incubation times, etc.

Where the negative selection step is repeated a plurality of times, each step or several steps may be performed in the presence of another agent which is not the co-factor. For example, where the co-factor is a certain protein, each step in the negative selection may be incubated in the presence in the medium of another non-co-factor protein being present in a different negative selection step.

It should be noted that by the above *in vitro* evolution method, proto-nucleozymes which are converted into nucleozymes by mere conformational change are eliminated. Such proto-nucleozymes can convert into a catalytically active form (i.e. into the nucleozyme) even spontaneously, without the co-factor, albeit at a low probability. Due to the unique and highly sensitive negative selection step employed in the above *in vitro* evolution method, all such proto-nucleozymes are identified and are removed, retaining only those proto-nucleozymes which have essentially no catalytic activity and need the co-factor, which complete the missing component, to become catalytically active.

The present invention will now be illustrated in the following non-limiting examples with an occasional reference to the annexed drawings:

5   **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows a schematic representation of a method of preparing a proto-nucleozyme of the invention, where the specific co-factor is a protein;

Fig. 2A, 2B and **2C** shows nucleic acid constructs which is a proto-nucleozyme where the co-factor is a nucleic acid sequence.

10   **Fig. 3** shows an improvement in the negative selection step of the method of Fig. 1;

Fig. 4A shows three dimensional structure of Group I intron ribozyme;

Fig. 4B shows the conserved nucleotide structure and secondary structure of Group I intron ribozyme;

15   **Fig. 4C** and **4D** show various modifications of the SunY ribozyme;

Fig. 4E shows various regions of SunY ribozyme;

Fig. 5 shows a comparison in ligation product yield (%) between different SunY constructs;

Figs. 6 and **7** show electrophoresis gels of the ligation products of
20   ribozymes in different experiments obtained by utilizing varying ratios of ribozymes to substrates taken at different times;

Fig. 8 shows a plot of the concentration ligation product (nM) created by a SunY P910 ribozyme at different ratios of ribozyme to the substrate as a function of time;

25   **Fig. 9** shows a double reciprocal (or Lineweaver Burt) plot based on the parameters of Fig. 8;

Fig. 10 shows the effects of various concentratins of SDS, GITC, urea and phenol (re-extracted with chloroform) or ribozyme reaction;

Fig. 11 shows the effect of varying concentrations of the PCR
30   inhibitors isopropanol, EtOH, urea, GITC and SDS on ribozyme reaction;

Fig. 12 shows the effect of phenol (either not extracted or re-extracted with chloroform) and a commercial specimen preparation kit, on ribozyme reaction;

Fig. 13 shows the effect of varying concentrations of GITC on ribozyme reaction (expansion of Fig. 10);

Fig. 14 shows the effect of varying concentrations of ATA, Spermidine, DMF, DMSO, Triton, Nonidet and Tween 40 on ribozyme reaction;

Fig. 15 shows the effect of varying concentrations of Spermidine, Triton X-100 and Tween 40 on ribozyme reaction (expansion of Fig. 14);

Fig. 16 shows the construct of the native SunY ribozyme;

Fig. 17 shows SunY construct in which fragment B (indicated in bold letters in the text) was replaced by 16s rRNA sequence of chlamydia;

Fig. 18 shows an electrophoresis gel indicating the ligation activity of various Group I intron chlamydia-based ribozymes;

Fig. 19 shows fluorescent labeled RNA constructs used as ribozyme substrates;

Fig. 20 shows the effect of denaturating PAGE after FRET monitoring;

Fig. 21 shows relative fluorescence as a function of time indicating ribozyme ligation activity in the presence of 5% SDS 100 mM urea or without additions;

Fig. 22 shows a schematic representatin of an RNA sequence pool which undergoes selection by *in vitro* evolution; and

Fig. 23 shows the ligation activity of proto-nucleotides which is cofactor (termed also *"efector"*) dependent.

In the description the following terms and definitions will be used:

Group I intron – An intron having a three-dimensional structure as depicted in Fig. 3A and a conserved nucleotide structure and secondary structure depicted in Fig. 3B.

6.2 – a substrate for SunY ligation having the sequence: 5' CCCUCU 3'.

P1 – shown in the right hand side of Fig. 4E

    P1 = when N is G

    P1RL = P1 as in Fig. 4E when N = 5' GGAGAAU 3'

P2 – shown in the ribozyme of Fig. 4E

    P2A =   A modification of P2 wherein the sequence 5' GGC UUA GAG AAG AAA UUC UUU AA 3' was inserted between # (–23) and 0

    P2H =   A modification of P2 wherein the sequence 5' GGU AA 3' was inserted between # (-5) and 0

P5 – shown in the ribozyme of Fig. 4E

    P5SA =   A modification of P5 wherein the sequence 5' AGC UAU A 3' was inserted between bases # 28 and # 32

    P5LA =   A modification of P5 wherein the sequence 5' AGC UAU AGA CAA GGC AAU CC 3' was inserted between bases # 28 and # 32

P9.0 – shown in the ribozyme of Fig. 4E

    P9L =   A modification of P9.0 with the sequence 5' AUU CUC 3'

    P910 =   A modification of P9.0 with the sequence 5' AUU CUC CAC C 3'

    P9A =   A modification of P9 with the sequence 5' AAA GCC AAU AGG CAG UAG CGA AAG CUG CGG 3'

        [substitution of P9 after the P9.0]

    P9JD =   A modification of P9 with the sequence 5' GGG GUG ACC CGA UC 3'

        [substitution of P9 after the P9.0]

F910 – a 10 base pair sequence derived from F9 having a sequence: 5' AUU CUC CAC C 3'.

    The following are a list of chimeras of various SunY constructs used herein:

Table 1

	Sun Y chimera name	Regions of SunY which were modified			
		P2	P5	P9.0	P9
		The modifying sequences			
1	SunY P9L	-	-	P9L	-
2	SunY P2P9L	P2A	-	P9L	-
3	SunY P910	-	-	P910	-
4	SunY P2P910	P2A	-	P910	-
5	SunY P2HP9L	P2H	-	P9L	-
6	SunY P9A	-	-	P9L	P9A
7	SunY P9JD	-	-	P9L	P9JD
8	SunY P3P9JD	P2A	-	P9L	P9JD
9	SunY P5SAP9L	-	P5SA	P9L	-
10	SunY P2P5SAP9L	P2A	P5SA	P9L	-
11	SunY P2HP5SAP9L	P2H	P5SA	P9L	-
12	SunY P5LAP9L	-	P5LA	P9L	-
13	SunY P2P5LAP9L	P2A	P5LA	P9L	-
14	SunY P2HP5LAP9L	P2H	P5LA	P9L	-

EXAMPLES

In the following Examples, the numbers in brackets indicate the number of the relevant step in the respective figure.

5   **Example 1   *In vitro* evolution where the co-factor is a protein**

i.   Preparing random panel of nucleic acid sequences (1)

A random panel of DNA sequences is prepared on a standard nucleic acid synthesizer using a program for generating random sequences.

A typical sequence is shown in Fig. 1 step (1) and comprises a
10   promoter (P) of about 20 b.p., a sequence which is cleavable in *cis* by a catalytically active ribozyme termed in the figure *"substrate"* (S) of about 10 b.p. (both these sequences are constant and not random), a random generated sequence (R) of 50-8000 b.p. averaging at 100, and two sequences, one upstream of the random sequence and one downstream
15   thereof, which serve as primer for PCR (M).

ii.   Preparation of immobilized nucleic acid sequences (2 and 3)

The DNA sequences of i. above, are transcribed, using a primer with biotin (B) at the 5' end (Fig. 1, step 2). The biotin, is then allowed
20   to react with avidin which is present on a solid support, such as Stepravidin beads (SA), so that each molecule of the random array becomes immobilized onto a solid support (Fig. 1, step 3).

iii.   Negative selection step (4 and 5)
25   A reaction mixture is added to the nucleic acid sequences prepared above which lacks the assayed co-factor protein but contains magnesium ions which are necessary for ribozyme catalytic activity. Alternatively, it is possible to add a reaction mixture which contains another protein (Pro') which is different than the intended specific co-factor
30   (Fig. 1, step 4a). Molecules which may be required for a catalytic activity such as Mg^{++} or GTP are then added to the reaction medium (Fig. 1, step

(step 4b(ii)) or possess a normal cleavage activity of ribozymes with the non–assayed co–factor (**Pro'**) (step 4b(i)) are released to the medium and are removed. After this negative selection only nucleic acid sequences which do not show any activity are maintained (step 4b(iii)). Thus the subsequent reaction mixture contains only these sequences (obtained in step 4b(iii)).

iv. Positive selection step (5)

The specific co–factor is protein (**Pro**) is then added to the reaction mixture (Fig. 1, step 5a).

Magnesium is then added to the reaction mixture in order to allow for the catalytic *cis* cleavage activity of the random nucleic acid sequences (Fig. 1, step 5b).

The nucleic acid sequences in the medium may be divided into three groups, as shown in step 5b:

Group i. proto–nucleozymes of the invention which are activated only upon formation of a catalytic complex with the specific co–factor protein;

Group ii. nucleic acid sequences, which complex with the protein co–factor, but do not feature any catalytic activity;

Group iii. (comprising the majority of the nucleic acid sequences) nucleic acid sequences which did not complex with the protein co–factor.

The last step of the positive selection comprises removal of those nucleic acid sequences which were freed from the immobilized beads (Group i.).

v. Amplification of separated sequence (6)

Sequences selected in iv. above are amplified. The amplified products are transcribed, bound to biotin and attached to a solid support as specified in (ii) above.

Steps (2) – (6) are then repeated for about 10–100 cycles, whereby the reaction mixture is gradually enriched with the proto-nucleozymes having the novel activity of the invention, i.e. that they are catalytically active only in the presence of the specific co-factor protein.

5   It should be noted that the positive selection step may precede the negative section step.

Example 2   Preparing co-factor binding haptens

As a preliminary step before constructing the DNA sequence of

10   step 1 (in Fig. 1), it is possible to find first short sequences (haptens) which are capable of binding the desired co-factor (protein).

The procedure for the preparation of such haptens is as follows:

1.   Short random sequences (about 80 base pairs) are prepared, and

15   allowed to react with the protein co-factor.

2.   Those sequences which bind the protein co-factor are identified for example, by the use of antibodies against the protein, by absorption of a protein to a membrane capable of absorbing proteins, etc. and are separated from the other sequences which did not bind to the protein.

20   3.   Sequences which were obtained in step 2 are amplified.

4.   Steps 2 and 3 above are repeated for 10–30 cycles so that the reaction mixture is enriched with haptens capable of binding the specific protein co-factor.

These short haptens then serve as part of the random sequence (R)

25   shown in step 1 of Fig. 1.

Example 3   Preparation of a semi-random nucleic acid sequences

Sometimes, for *in vitro* evolution purposes, it is desired that the newly prepared panel of sequences resemble to some degree a known

30   sequence, for example that of a ribozyme. If for example, it is desired that the semi-random sequence has a 70% average similarly to a specific nucleic

of four pure solutions containing each of the nucleotides (A, T, G and C), with four bottles each containing a solution composed of 70% one nucleotide (for example A), and 10% of each of the other three nucleotides (10% G, 10% T and 10% C). By instructing the synthesizer to construct a

5   sequence utilizing these four mixtures, it is possible to obtain a sequence which has a 70% similarity to a known sequence. Other percentages of similarity may of course be used.

Example 4   *In vitro* evolution where the co-factor is a nucleic acid
10   **sequence**

I.   **A proto-nucleozyme for use in the detection of a short nucleic acid sequence co-factor (Fig. 2A):**
15

A known ribozyme sequence is chosen having a specific core region (C). The core region is screened for the sequences which most resemble the nucleic acid co-factor. The resembling sequences are removed

20 altogether (Fig. 2, step 2a), or are replaced by a completely random sequence (R) (Fig. 2, step 2b).

The sequences upstream and downstream of the missing core region or the random region are fitted with two flanking sequences (F) which are complementary to the sequences which flank the intended co-

25 factor (step 3(a) and (b)). The original ribozyme sequences of the ribozyme obtained by step 3(b) are then doped, i.e. to become semi-random (R') as explained in Example 3 (step 4(b)), for example by having a 70% similarity to the original ribozyme sequence.

As regards the two parts of the precursor obtained in step 3(a),

30 only the original ribozyme sequence of one part is made to be semi-random (R') while the other part remains constant (step 4(a)).

The sequences obtained in 4(a) or 4(b) may then undergo the same steps of *in vitro* evolution of Example I (ii)-(v).

Alternatively, it is possible to construct a sequence comprising from $5' \to 3'$; a completely random sequence (**R**), a flanking sequence (**F**) and a semi-random sequence (**R'**) having a 70% similarity to a known ribozyme (Fig. 2B) or the same sequence in the $3' \to 5'$ orientation (Fig. 2).

5

II. A proto-nucleozyme for use with detection of long nucleic acid sequence co-factors

The procedure of Example 4(I) above is essentially repeated,
10 however, without inserting the flanking sequences (F) in the sequence. Since the co-factor to be detected is itself a very long nucleic acid sequence (for example ribosomal RNA), it is assumed that even without manipulation, some of the sequences of the long co-factor will be complementary (to some extent) to sequences of the core which have not been removed or
15 replaced by random sequences.

III A proto-nucleozyme for use in the detection of a nucleic acid sequence co-factor which is unknown

20 At times, it is not *a priori* known exactly what is the sequence of the target co-factor, for example, it may be one of many sequences of a virus or a bacteria the presence of which is to be detected. In such a case, it is best to randomly remove some sequences from the core of a ribozyme, and then render the remaining parts semi-random. It is desired that the
25 removed sequences are completed by the sequences of the unknown bacteria.

When repeating steps (ii)–(v) of Example 1 with the construct prepared as above, it is necessary that in the negative selection, step (iii), the sequences of viruses or bacteria which are added to the reaction medium are similar to the one to be detected, in order to eliminate any nucleic acid
30 sequences which are activated by other viruses or bacteria. In the repeating cycles, other types of viruses or bacteria may be added for the negative selection step.

Example 5   Improvement in negative selection step using a tag sequence

i.   Preparing random panel of nucleic acid sequences (1)

5   A panel of DNA sequences is prepared on a standard nucleic acid synthesizer using a program for generating desired sequences.

A typical sequence is shown in Fig. 3 step (1) and comprises a promoter (P) of about 20 bases, a substrate S which can be cleaved in *cis* by a catalytically active oligonucleotide, a first primer for PCR amplification

10   (PCR1), a first restriction site C_1 of about 4-8 bases, a random tag (TAG) sequence of about 15 bases, a second restriction site C_2 of about 4-8 bases (both these sequences are constant and not random), a variable sequence (V) of 50-8000 b.p. averaging at 100, said variable sequence being a candidate capable of evolving to a functional sequence. The variable sequence may

15   be similar to variable sequences of other oligonucleotide species, for example since all sequences are dopped, i.e. having a certain percent of nucleic acids identical with those of a known ribozyme and a certain percentage of randomness; and a second PCR primer sequence (PCR2). The variable sequence is a candidate for evolving to a sequence which after

20   complexation with a specific co-factor, which completes a missing component becomes catalytically active.

ii.   Preparation of immobilized nucleic acid sequences (2 and 3)

The DNA sequences of i. above, are transcribed, using a primer

25   with biotin (B) at the 5' end (Fig. 3, step 2). The biotin, is then allowed to react with avidin which is present on a solid support, such as Strepravidin beads (SA), so that each oligonucleotide of the panel becomes immobilized onto a solid support (Fig. 3, step 3).

iii. Positive selection step (4)

The specific assayed agent which is a protein (serving as the selected set of conditions) (**Pro**) is then added to the reaction mixture (Fig. 3, step 4(a)).

5   Magnesium and/or other co-factors required for catalytic activity, are then added to the reaction mixture in order to allow for the catalytic *cis* cleavage activity of the variable sequences of the oligonucleotides on substrate sequence S (Fig. 3, step 4(a)).

The oligonucleotide sequences in the medium may be divided into

10   three classes, as shown in step 4(b):

Class i.   oligonucleotides activated only upon formation of a catalytic complex with the specific co-factor **Pro** and cleaved substrate S and are thus released to the medium;

Class ii.   oligonucleotides, which feature catalytic activity even

15   without a protein;

Class iii.   (comprising the majority of the nucleic acid sequences) oligonucleotides which did not feature catalytic activity in the present co-factor.

The last step of the positive selection comprises collecting of

20   those oligonucleotides which were freed from the immobilized beads (Class i and Class ii).

iv. Amplification of separated sequence (5)

If desired, protein attached to the oligonucleotides can be removed

25   by denaturation through heating or by phenol–chloroform extraction.

Sequences collected in step 4 above are reversed transcribed and amplified by PCR. Since substrate sequence S was cleaved (step 4b) this sequence should be reconstructed by using, either for reverse transcription or for PCR proposes primers which contain the sequence of the cleaved

30   substrate and tus the amplified and reconstructed product is again identical to 1. The amplified products are transcribed, bound to biotin and attached

v.   **Negative selection step (7)**

The amplified and immobilized oligonucleotides of step 6 are subjected to a negative selection step. In this step the catalytic activity is determined either in the absence of any protein, or in the presence of non-
5   co-factor agents, similar to the specific co-factor which is added to the reaction mixture, the agents being a protein similar to the specific co-factor (**Pro'**) (step 7a); magnesium and/or other reagents required for catalytic activity are added (step 7b).

The oligonucleotides released to the medium by cleavage of the
10   substrate sequence S belong to three classes:

Class i.   in which the non-co-factor protein brought about cleavage of substrate sequence;

Class ii.   which includes oligonucleotides cleaved with no need for any external co-factor whatsoever; and

15   Class iii. which are not cleaved at all.

Both Classes i. and ii. are collected (step 7c) and the protein is removed (step 7d). The sequences are reversed transcribed and amplified by PCR in order to produce a double-stranded construct (step 8). Suitable restriction enzymes are added in order to cleave sequences C_1 and C_2, one
20   cleavage site forming a blunt end and one a sticky end (step 9). The sticky end is completed with the aid of biotinated nucleotides and then the separated TAG sequence is immobilized on a solid support (avidin) and denaturated so that there remains a single-stranded denaturated immobilized tag sequence.

25   The immobilized tag sequences of step 10 are brought into contact with oligonucleotides collected of step 4(b) (Class i and Class ii) (step 11). Molecules of said group which hybridize with the immobilized TAG sequence are removed (step 12) so there remain only molecules which feature cleavage in the presence of the specific co-factor (**Pro**) and do not
30   feature cleavage activity without any protein or in the presence of a non-co-factor agent (**Pro'**).

The oligonucleotides obtained in step 11 are subjected again to all preceding steps for 2-1000 cycles, preferably 10-100 cycles, most preferably 20-30 cycles.

5   **Example 6   Ligation of substrates PIRL and 6.2: Comparison between 15 different SunY Ribozymes**

A comparison of the ability to ligate the substrates PIRL and 6.2 was carried out between the following 15 SunY-derived ribozymes:

10

■	SNYP9L;	■	SNYP2AP9JD;
■	SNYP2P9L;	□	SNYP5SAP9L;
□	SNYP910;	■	SNYP2P5SAP9L;
□	SNYP2P910;	■	SNYP2HP5SAP9L;
■	SNYP2HP9L;	■	SNYP5LAP9L;
■	SNYP2AP9A (up);	■	SNYP2P5LAP9L;
□	SNYP2AP9A (down);	■	SNYP2HP5LAP9L;
■	SNYP9JD;	■	SNYP9L

15

20

The Reaction Conditions were as follows:

Molar Ratio Ribozyme: PIRL:6.2 = 1:5:5

25   Final Ribozyme Concentration: 0.5 μM.

The reaction mixture containing the ribozyme, PIRL, and the 6.2 substrate was preincubated at 58°C for 2 minutes in a buffer containing 30 mM tris-HCl, pH 7.4 (at 25°C)/10 mM NH$_4$Cl/0.4 M KCl/20% ethanol. The solution was cooled to 45°C and the reaction started by the addition of

30   90 mM final MgCl$_2$ in a reaction volume of 5 ul. The reaction was stopped after 30 minutes by the adition of 10 ul 2x loading buffer containing 7 M urea. Samples were heated for 2-3 minutes at 80°C and loaded onto a 20% PAA gel containing 7 M urea. The radioactive gels were analyzed with the aid of a Biorad Phosphoimager System.

The results are shown in Fig. 5. As can be seen, various ribozymes which were derived from the SunY ribozyme maintain the ability to ligate substrates.

5   **Example 7   Kinetic Studies on ribozymes SunY P910 using the substrates PIRL and 6.2**

Ribozyme SunY P910 was used for ligation of the PIRL and 6.2 substrates.

10   The Reaction Conditions were as follows:

Molar Ratios between Ribozyme: PIRL:6.2 = 1:2.5:2.5 to 1:20:20

Final Ribozyme Concentration: 0.25 μM.

The reaction mixture containing the ribozyme and the substrates PIRL and the 6.2 was preincubated at 58°C for 2 mins. in a buffer

15   containing 30 mM tri-HCl pH 7.4 (at 25°C)/10 mM NH$_4$Cl/0.4 M KCl/20% ethanol. The solution was cooled to 45°C and the reaction started by the addition of 90 mM final MgCl$_2$ concentration in a final reaction volume of 5 μl.

At the designated times the reactions were stopped by the addition

20   of 10 μl 2x loading buffer containing 7 M urea. Samples were heated for 2-3 minutes at 80°C and loaded onto a 20% PAA gel containing 7 M urea.

The radioactive gels were analyzed in a Biorad Phosphoimager system. The results are shown in Figs. 6 and 7.

These results were used to plot kinetic graph depicted in Fig. 8,

25   which was used to create Lineweaver-Burt Plot of Fig. 9. The calculated Km was 4.7 μm and the calculated K_{cat} was 6 min^{-1}. These results indicate a vast improvement in Km and a 1800 improvement in K_{cat} as compared to results obtained previously (data not shown) with the native SunY ribozyme.

30   **Example 8   Effect of lysis conditions on ribozyme kinetics**

Conventional amplification systems are based on protein enzymes. Therefore, either tedious purification steps are necessary or very special and

mild lysis conditions have to be worked out. In contract, ribozyme reactions are very robust and insensitive to harsh conditions, like:

- high concentrations of the strong, ionic detergent SDA (up to the limit of solubility: 5% at 45°C in the presence of 400 mM KCl)

5
- high concentrations of the nonionic detergent Triton X-100 (10% at 45°C are even stimulatory)

- denaturing agents like urea (100 mM has no effect)

- the nuclease inhibitor EDTA

- the ribonuclease inhibitor ATA

10
- organic solvents like isopropanol, ethanol.

The effects of all additives on ribozyme reactions were analyzed by polyacrylamide gel electrophoresis and are shown hereinbelow.

I. **Effect of denaturating agents (SDA, GITC, urea and phenol) on**
15 **ribozyme reaction rate**

Reaction conditions:

0.5 μM and 5 μM P1 BS and 5 μM ^{32}P labeled 6.2 substrate were incubated at 58°C in reaction buffer containing SDS, GITC, urea or
20 chloroform in concentrations indicated in Fig. 9 or 25% (v/v) of the aqueous supernatant above the phenol or phenol/chloroform phase. The arrow at the top of the figure indicates a re-extraction of the aqueous phase with chloroform; again 25% of the aqueous supernatant was added to the ribozyme reaction. After slow cooling down to 45°C the reaction was
25 started by the addition of $MgCl_2$ to a final concentration of 150 mM. After 60 mins. the reaction was stopped by quenching with 8 M urea.

The results are shown in Fig. 10. As can be seen, addition of denaturing agents or phenol after re-extraction with chloroform did not significantly change the ribozyme as compared to control.

30

II Effect of PCR inhibitors (isopropanol, EtOH, Urea, GITC and SDS) on ribozyme reaction rate

0.5 μM SunY and 5 μM ^{32}P labeled 6.2 substrate and 5 μM PI BS
5   were preincubated at 58°C in reaction buffer containing the indicated concentrations of various PCR inhibitors (EtOH as indicated, all others contain 10% EtOH) and slowly cooled down. After the reaction reached 45°C, the reaction was started by addition of $MgCl_2$ to a final concentration of 150 mM. After 60 mins. the reaction was stopped by quenching with
10   8 M urea. The results are shown in Fig. 11.

As can be seen, various agents which inhibit PCR reactors do not significantly effect ribozyme-based reactions, thus making ribozye-based reaction agents more attractive for detection purposes.

15   ## III Effect of phenol and commercial speciman preparation kit on ribozyme reaction rate

0.5 μM SunY and 5 μM PI BS and 5 μM ^{32}P-PIP were preincubated at 58°C in reaction buffer containing the following additives:
20   25% (v/v) of the aqueous layer above phenol or phenol/chloroform. The arrow indicates a re-extraction with chloroform; again 25% of the aqueous layer was added. The following solutions were added from the commerical Amplicor CT/NG kit (*Chlamydia trachomatia/Nelsseria gonorrhoeae*) Specimen Prep Kit (Roche): 25% or 10% (v/v) of Urine Wash Buffer; 25%
25   or 10% of CT/NG Specimen Diluent; 25%, 10% or 8.3% of CT/NG Lysis Buffer which contains an unspecified, nonionic detergent.

After the temperature reached 45°C, the reaction was started by the addition of $MgCl_2$ to a final concentration of 150 mM. The reaction was stopped after 60 mins. by quenching with 8 M urea. The results are shown
30   in Fig. 12.

As can be seen, both phenol, which is used heavily in various laboratories for specimen preparation, (after re-extracted by chloroform) and a commercially available specimen preparation kit did not effect the ribozyme reaction. Laboratory procedure carried out in the presence of

phenol should include a re-extraction step by chloroform before submitting the specimen to ribozyme reaction.

IV Effect of varying concentrations of GITC on ribozyme reaction rate

0.25 μM SunY and 5 μM ^{32}P–labeled 6.2 substrates and 5 μM PI BS were preincubated at 58°C in reaction buffer containing GITC in 100 mM incrementa from 0 to 1 M and slowly cooled down. After the temperature reached 45°C, the reaction was started by addition of MgCl$_2$ to10 a final concentration of 150 mM. After 30 mins. the reaction was stopped by quenching with 8 M urea.

The results are shown in Fig. 13. As can be seen up to 300 mM GITC did not effect the reaction rate.

V Effect of various additives (ATA, Spermidine, DMF, DMSO, Triton, Nonidet and Tween 40) on ribozyme reaction

0.5 μM SunY and 5 μM PI BS and 5 μM ^{32}P–labeled 6.2 substrates were incubated at 58°C in reaction buffer containing the indicated additives. After slow cooling to 45°C, the reaction was started by the addition of MgCl$_2$ to a final concentration of 150 mM. After 60 mins. the reaction was stopped by quenching with 8 M urea. Relative product yields are indicated: the value for the control without additive was set to 100. The significant stimulation with Triton X-100 is printed in bold.

The results are shown in Fig. 14. As can be seen, most of the additives did not significantly reduce the ribozyme reaction while Triton X-100 increase ribozyme activity by 80%.

VI Effect of additives (Spermidine, Triton X-100 and Tween 40) on reaction rates

0.5 μM SunY and 5 μM PI PS and 5 μ ^{32}P–labeled 6.2 substrates were incubated at 58°C in reaction buffer containing the additives indicated

addition of $MgCl_2$ to a final concentration of 150 mM. After 60 mins. the reaction was stopped by quenching with 8 M urea. Relative product yields are indicated: the value for the control without additive was set to 100. The significant stimulation with Triton X-100 is printed in bold. The results are shown in Fig. 15.

As can be seen from the additives used, only Tween 40 at a concentration of 20% significantly inhibited the reaction. Triton X-100 at all concentrations tested was able to increase activity by 28% to 85%.

Example 9   Chalamydia-based proto-nucleozymes

Part of the catalytic core of an evolved SunY ribozyme was substituted with 16s rRNA sequences from *Chlamydia trachomatis*. Corresponding changes were introduced in the ribozyme to accommodate the Chlamydia substitution. Fig. 16 shows the wild type ribozyme with the sequence to be substituted in bold print. 17 different ribozyme sequences were tested for splicing activity. Fig. 17 shows the actual sequence of the ribozyme (option 29) which is active when part of its core sequence was substituted by chlamydia sequences (the latter represented in bold).

The experiments were conducted as follows:

In Fig. 18, 2 μM of each of the 17 putative Chlamydia dependent ribozymes were reacted with 4 μM of substrate for 18 hours at 45°C. The wild type SunY ribozyme was used as positive control. Reactions were stopped with 5 mM EDTA and 3.5 M urea, final concentration, and then run on a 20% PAA-7M urea gel. The results are shown in Fig. 18.

These results show that a ribozyme (option 29) where part of its nucletoides were replaced by 16s rRNA sequences of *Chlamydia trachomatis* can be catalytically active. This ribozyme without the *Chlamydia* sequence can serve as a proto-nucleozyme of the invention and used in an asay to detect the presence of chlamydia, since the *Chlamydia* sequences (serving as co-factors) can complete its missing sequences and thus form a catalytically active nucleozyme.

Example 10   Detection of ribozyme ligation reaction utilizing fluoresence energy transfer (FRET)

A convenient model for detecting ribozyme activity, which may
5   be cleavage, splicing or ligation is to use a fluorescent dye such as
fluorescein (F) and a quencher dye CY-5. When the two dyes are brought
together (for example, due to ligation or splicing), the reproter dye (F) can
absorb light, but fluorescence is quenched by FRET: the energy is
transmitted to the quencher dye CY-5 which emits its distinctly diferent
10   fluorescence light at 670 nm.   As a consequence of the ligation or splicing
reaction, the F-labeled nucleotide(s) is/are liberated. Now, the reporter dye
can emit its own, characteristic fluorescence at 522 nm.

Two small RNAs which are well characterized substrates for
ligation by Group I ribozymes, were chosen.
15   Each RNA is labeled with only one dye:

-    the large RNA carries the *"reporter"*dye fluorescein (F) at its 5'-end;

-    the small RNA carries the *"quencher"* dye CY-5 at its 5'-end.

Both RNAs associate under reaction conditions.

The ribozyme constructs are shown in Fig. 19.
20   Before the ribozyme reaction, both RNA substrates hybridize to
form a nicked hairpin. Incoming light (hv; represented in the figure as a
solid arrow) is absorbed by fluoresceing (F) and transferred to CY-5
(dashed arrow) which subsequently emits its own characteristic fluorescence
at 670 nM (hv; zigzag arrow).
25   After the reaction, the fluorescein-labeled trinucleotide (F-UGG)
is liberted and the absorbed light is emitted directly from fluorescein with
the characteristic wavelength of 522 nm.

Diagram 11:   An Example of Real Time monitoring

30   FRET can be measured by calculating the ratio of the signal from
the reporter dye F (at 522 nm) to an inert signal at 500 nm.

This ratio is plotted versus the time axis: After 5 minutes

Each measurement is evident by a small peak: due to photo-bleaching, the signal drops during the measuring period and recovers during the dark interval. The results are shown in Fig. 21.

5   Reaction condtions: 1 μM SunY and 1 μM 5'-fluorescein-labeled PI BS and 5 μM 5'-CY-5-labeled PIP were incubated in the ABI Prism 7700 for real time monitoring. Incubation at 45°C was performed for 180 mins. with data collection in 5 minute intervals. Change of the relative fluorescence of the reporter dye fluorescein is shown. For further analysis, 10 μl aliquots

10   of the samples were analyzed by denaturing polyacrylamide gel electropho-resis (shown in Fig. 19). The comparison shows that GITC quenches the fluorescein signal, but does not inhbit the ribozyme reaction.

The results are shown in Fig. 20 which shows also the effect of denaturants on reaction rate and detection.

15   In the absence of ribozyme, there is no change of the FRET signal. In the presence of ribozyme, without further additives, the signal changes from an initial value "4.2" to the final value "6.2".

Very similar changes were observed, if 200 mM urea or 5% SDS have been added, indicating that these additives do nto change significantly

20   the reaction rate nor the detectable signal.

Although 100 mM guanidinium isothiocyanate (GITC) does not inhibit the ribozyme reaction (Fig. 12), it is impossible to include GITC if fluorescein is used as reporter dye since GITC is a very strong quencher for fluorescein. However the effect of GITC is specific to the dye since no

25   quenching was observed with other fluorochromes like CY-5 (data now shown).

Example 12

I.   **Production of target dependent ribozyme proto-nucleozymes from**
30   **a random sequence**

In vitro evolution was used to prepare proto-nucleotzymes which

The selection scheme is similar to that employed by Bartel and Szotstak (*Science*, **261**:1411–1418 (1993)). A random sequence RNA pool (N90) begins with a 5' triphosphate. The pool is captured on an affinity matrix complementary to the 3' end of the pool and mixed with a RNA

5   substrate tagged with a DNA sequence. Following ligation, the RNA can be eluted from the column, reverse-transcribed via a cDNA primer, and PCR-amplified via a primer that is complementary to the newly ligated DNA tag. A nested PCR reaction using an internal primer generates a template that can again be transcribed to regenerate selected ligases. This

10   cycle can be used for selection of the fastest and most active ligases.

The schematic pool of RNA sequences on which selection is carried out is shown in Fig. 22, together with the sequence of its 3' tail and its complementary cDNA primer.

After several rounds of selection the pool was cloned and

15   individual clones were assayed, for activity and dependence on co-factor sequence.

The major class of selected ligases can efficiently catalyze the addition of the mixed labeled RNA-DNA substrate to themselves. A time course was carried out under standard assay conditions, and ligation products

20   were separated from unligated ribozymes by separation on a denaturing PAA gel. In the absence of either Mg^{+2} or the co-factor, which was a specific nucleic acid sequence, no reaction was observed. The relative amount of radioactive label in ligated and unligated ribozymes was determined using a Phosphoimager, background in the absence of co-factor was subtracted

25   from these values, and the extent of reaction was plotted as a function of time. Initial rates and the full extent of the reactions were determined using standard curve-fitting procedures, and are shown in Fig. 23. As can be seen in the absence of co-factor or Mg^{+2} no ligated products was produced.

The clones were further examined for specificity. A series of

30   DNA or RNA nucleotide sequence co-factors was synthesized and their abilities to activate the proto-nucleozymes were determined. Values were

relative to background. Experiments carried out in duplicate or triplicate indicated that the relative activities of *"active"* (e.g. lines (1) and (9)) and *"inactive"* (e.g. lines (6) and (10)) co-factors are consistent between experiments.

5   The results are shown in Table III.

Table III – Specificity of the co-factor–Dependent Ribozyme

Terminal guide sequence of ribozymes

Co-factor sequence

	...	GG	UGC	CUC	GUG	AUG	UCC	AGU	CGC	ACG	3'	Type	relative activity (ribozyme)
1				GAG	CAC	TAC	AGG	TCA	GCG		5'	wild type	1.0
2			G	GAG	CAC	TAC	AGG	TCA	GCG		5'	Extended (+1)	0.09
3				AG	CAC	TAC	AGG	TCA	GCG	T	5'	receded (−1)	–
4				AAG	CAC	TAC	AGG	TCA	GCG		5'	C:A mismatch	0.08
5				AAG	CAC	TAC	AGG	TCA	GCG		5'	fully complementary	1.1
6				GAG	CAC	TAC	AGG	TCA	GCG		5'	dideoxy terminal	–
7				GAG	CAC	UAC	AGG	UCA	GCG		5'	wild type RNA	0.72
8				GAG	CAT	TAC	AGG	TCA	GCG		5'	G:T wobble	1.0
9				GAG	CAC	TAC	AGG				5'	wild type (12)	0.44
10				GAA	CAC	TAC	AGG				5'	C:A mismatch	–

TGS transition mutant ribozyme

Terminal guide sequence of ribozymes

Co-factor sequence

	...	GG	UGC	CCU	ACA	GCA	CUU	GAC	UAU	3'	Type
11				GAG	CAC	TAC	AGG	TCA	GCG	5'	wild type	–
12				GGA	TGT	CGT	GAA	CTG	ATA	5'	fully complementary	1.3

A variety of potential co-factors were synthesized and assayed them for their ability to enhance ligase activity; the results of these experiments are shown in Table III. Oligonucleotide co-factors that extended complementarity towards the 5' end of the RNA or that receded

5   towards the 3' end showed little or no activation relative to no effector controls (Table III, lines (2) and (3)). These results seemed to indicate that the position of the co-factor was essential for activity, and that the 3' end of the co-factor might play a role in activation. A co-factor was synthesized with a G to A substitution at the 3' terminus. The C:A mismatch was

10   much less active than the fully complementary effector (line (4)). However, the loss of activity seen with the G to A change in the effector could be suppressed by a compensatory C to U change in the ribozyme (line (5)). These results seemd to indicate that the 3' end of the ribozyme was acting like a "terminal guide sequence" (TGS). The co-factor that terminated in

15   a dideoxy nucleotide could not activate catalysis (line (6)). In contrast, an all-RNA co-factor activated catalysis nearly as well (relative activity = 0.72; line (7)) as the original all-DNA effector. Taken together, these results emphasize that the ribozyme is capable of specific recognition of its specific oligonucleotide co-factor.

20

II. Activation in the presence of random sequences

The experiment described in I above was repeated with a co-factor of 12 mer in the presence of a random sequence of 12 nucleotide, or in the presence of both the random sequence and the co-factor, either when

25   both are at the same concentration (1.3 μM) or where the random sequence is at an excess (10:1).

The results are shown in Fig. 23. As can be seen a 12 mer co-factor was able to activate the proto-nucleozyme at several concentrations while the random sequence pool showed no such activation. Moreover, the

30   random sequence pool, even in excess concentration does not interfere with the ability of the correct co-factor to activate the proto-nucleozyme. These results show the specificity of the proto-nucleozyme of the invention to its

specific co-factor and demonstrate that the presence of other sequences does not interfere with this specificity.

CLAIMS:

1.   A proto–nucleozyme, being a nucleic acid molecule or a complex of nucleic acid molecules, having essentially no catalytic activity but can complex with a co–factor to form a nucleozyme which possesses a catalytic activity, the proto–nucleozyme lacks a component essential for the catalytic activity of the nucleozyme and said co–factor provides said component.

2.   A proto–nucleozyme according to Claim 1, wherein said missing component is a missing segment of one or more nucleotides or a missing link between two nucleotides.

3.   A proto–nucleozyme according to Claim 1 or 2, wherein the nucleozyme is a ribozyme.

4.   A proto–nucleozyme according to Claim 1 or 2, being a product of *in vitro* evolution.

5.   A proto–nucleozyme according to any one of Claims 1 to 4, wherein said specific co–factor is a nucleic acid sequence.

6.   A method for the detection of an agent in a medium which comprises the following steps:

(a)   contacting said medium with a proto–nucleozyme of any one of Claims 1-4, wherein said agent is the specific co–factor required for the formation of said catalytic complex;

(b)   providing or maintaining conditions allowing catalytic activity of the catalytic complex; and

(c)   assaying for the presence of products of the catalytic activity in the medium, such presence indicating the presence of said agent in the medium.

7.   A method according to Claim 6, wherein said catalytic activity is cleavage.

8.   A method according to Claim 6, wherein the catalytic activity is ligation.

9.　　　　An *in vitro* evolution method for the production of a proto-nucleozyme of Claim 1, the method comprising:

(a)　preparing a panel of different nucleic acid molecules, at least part of the sequence in the molecules of the panel is a random or a partially random sequence, such that said part has a different sequence in different molecules of the panel;

(b)　adding said co-factor to said panel and incubating under conditions permitting said catalytic activity;

(c)　separating between the nucleic acid molecule of said panel which feature said catalytic activity and such which do not obtain a first selected panel of nucleic acid molecules which feature said catalytic activity;

(d)　amplifying the nucleic acid molecules of said first selected panel;

(e)　incubating said first selected panel with a reaction mixture, being devoid of said co-factor, under conditions permitting said catalytic activity;

(f)　removing nucleic acid molecules which featured catalytic activity, thereby obtaining a second selected panel of nucleic acid molecules devoid of the removed nucleic acid molecules; and

(g)　repeating steps (b)-(f) over a plurality of cycles, to obtain said proto-nucleozyme.

10.　　　　A method according to Claim 9, wherein said cycles are repeated 10-100 times.

11.　　　　A method according to any one of Claims 9 or 10, wherein the molecules of the panel of nucleic acid molecules in step (a) have a sequence which is derived from a known nucleozyme sequence with some proportion of the nucleotides being replaced by other nucleotides.

12.　　　　A method according to Claim 10, wherein said some proportion is about 1-30%.

13.　　　　A method according to Claim 11 or 12, comprising at least one amended cycle, wherein the amplification step of the molecules of the

selected panel comprises also replacing a small proportion of the nucleotides in the molecules of said selected panel.

14. A method according to Claim 13, comprising a plurality of such amended cycles.

5   **15.** An *in vitro* evolution method for the production of the proto-nucleozyme of Claim 1, which nucleozyme together with a co-factor from a catalytic complex having a catalytic activity imparted by a functional sequence of said proto-nucleozyme, the method comprising:

(a) preparing a mixture of different oligonucleotide candidates for 10 evolving to said proto-nucleozyme each of which comprises a variable sequence being a candidate for evolving in said functional sequence and a tag sequence, each of the two sequences being different than corresponding sequences of different oligonucleotides in the mixture, the variable sequence and the tag sequence being linked by at least one cleavable sequence;

15   (b) processing the mixture through positive and negative selection steps, each step optionally followed by an oligonucleotide amplification step, there being at least one positive selection step and at least one negative selection step, these steps comprising:

(ba) a positive selection step comprising applying said specific 20 co-factor and separating between the oligonucleotides which have and those which do not have catalytic activity to obtain a first selected mixture comprising a first group of oligonucleotides featuring catalytic activity in the presence of the co-factor;

(bb) an amplification step comprising amplifying said first group 25 of oligonucleotides in said first selected mixture to produce a plurality of copies of each of said first group of oligonucleotides to obtain a first amplified mixture;

(bc) a negative selection step comprising:

(bca) applying a reaction mixture devoid of the specific 30 co-factor and separating between a second group of oligo-nucleotides which do not have catalytic activity in the absence of the co-factor and a third group of oligo-

nucleotides having the catalytic activity in the absence of the co-factor, to obtain a second selected mixture comprising said second group of oligonucleotides and a third selected mixture comprising said third group of oligonucleotides;

(bcb)   amplifying said third group of oligonucleotides in said third selected mixture to produce a plurality of copies of each of said third group of oligonucleotides to obtain a second amplified mixture;

(bcc)   cleaving the cleavable sequence of the oligonucleotides in the second amplified mixture, separating between the variable and the tag sequences and collecting the tag sequences;

(bcd)   contacting the collected tag sequences with the second selected mixture under stringent conditions of hybridization and removing hybridized oligonucleotides from other oligonucleotides of the second mixture, thereby obtaining a fourth selected mixture of oligonucleotides essentially devoid of oligonucleotides which have catalytic activity in the absence of the co-factor;

where said positive selection step precedes said negative selection step, said positive selection step is applied on said mixture prepared in step (a) and said negative selection step is applied on said first amplified mixture; and where said negative selection step precedes said positive selection step, said negative selection step is applied on said mixture obtained in step (a) and said positive selection step is applied on said fourth mixture.

16.   A method for selectively destroying a specific cell population, containing or expressing a specific agent, the method comprising:

(a)   providing a proto-nucleozyme of Claim 1, which when present in the catalytic complex has a catalytic activity which is cytotoxic or produces cytotoxic to the cells or has reaction products, and wherein

(b) inserting said proto-nucleozyme into cells containing or expressing said agent, or applying said proto-nucleozymes to tissue, suspected of containing a population of cells containing or expressing said agent, under conditions or using a vehicle so as to insert said proto-

5 nucleozyme into said cells.

17. A method for inhibiting expression of undesired genes in cells, comprising:

(a) providing a proto-nucleozyme of Claim 1, wherein said nucleozyme has a catalytic activity which is targeted at a specific nucleic acid

10 sequence or gene expression product so that once active within a cell, the undesired gene expression will be inhibited;

(b) inserting said proto-nucleozyme into cells containing or expressing said gene, or applying said proto-nucleozyme to tissues suspected of having a population of cells containing or expressing said gene, under

15 conditions or using a vehicle so as to insert said proto-nucleozyme into said cells.

18. A method according to Claim 17, wherein said co-factor of the proto-nucleozyme is either a nucleic acid sequence of said gene or a transcription product of the gene.

20 19. A pharmaceutical composition comprising a proto-nucleozyme according to any one of Claims 1-5 and a pharmaceutically acceptable carrier.

20. A pharmaceutical composition according to Claim 19, wherein said carrier comprises liposomes.

1)

P S M R M

2) β

3) SA β

4) (a)

SA β

SA β + Pro'

SA β

↓ + Mg⁺⁻

(i) SA β

Pro'

4) (b) (ii) SA β ⟹ remove

(iii) SA β

5) (a)

+ Mg^{++}

5) (b)

(i) SA β

(ii) SA β   Pro   } remove

(iii) SA β

Pro } obtain

6) PCR

F i g . 1 continue

F i g . 2 A

R   F   R'

Fig.2B

R'   F   R

Fig.2C

1)

S PCR₁ C₁ TAG C₂   V   PCR₂

3' P   5'

T7 POLYMERASE
BIOTINATED PRIMER

2)   Ⓑ ～～～～～～

3)   SA Ⓑ ～～～～～～

2)   (a)   +   PRO
                                Mc

      (b) (i)   SA Ⓑ ～～   ～～～ PRO ～～ ⎫
                                              ⎬ COLLECT
           (ii) SA Ⓑ ～～   ～～～～～   ⎭

REMOVE —(ii)   SA Ⓑ ～～～～～～

5)   DENATURATION OF PROTEIN
     REVERSE TRANSCRIPTION
     PCR

5)   TRANSCRIPTION+IMMOBILIZATION

     SA Ⓑ ～～～～～～

     SA Ⓑ ～～～～～～

Fig. 3A

7)   (a)   ↓   +   PRO

        (b) Mg^{++}

(c)   (i)   SA B $\sim$ PRO' $\sim$ } COLLECT

     (ii)   SA B $\sim$ $\sim\sim$

REMOVE ←—(ii)   SA B $\sim\sim\sim$

(d)   DENATURATION OF PROTEIN
        PCR

                   ↓

8)   ———— TAG ————
        ————————————

          ↓   RESTRICTION
             ENZYNES

9)   —— —— —TAG— ——
      —— —— ———— ——

          ↓   BIOTINATED NUCLEOTIDES
              ANNEALING

10)   SA B — TAG ——

11)   ↓   +   CONTACT WITH
                OLISONUCLEOTIDES

               SA B   TAG

12) $\sim\sim$ ‖‖‖‖ $\sim\sim$ } REMOVE

Fig. 3B $\sim\sim\sim\sim\sim\sim$ } MAINTAIN

Fig. 4A

Fig. 4B

P9 of the SunY ribozyme as substrate

```
                         G
                     A     C
                     C : G
                     U : A
                     C : G
                     U : A
                     C       C
                     A
                     A       A
                     A       A
                     G : U
                     G : C
                     G : C
                     G : C
                     C : G       UAAAU      A
5'···GAAAA·UCUGCC·UAA··  A:U···GC        CAGC  A
            : : : :                      :: : |
         AGAUGG                 CG       GUCG U
         |            C       C  UAAAU      A
         |            U             U
         |            C             A
      U·CUCAAC          AUCAGCA
   A   : : : : : :       : : : : : :
      A·GAGUUG··AAGGUA··UAGUC·U·A·AA····C
                                        U
       SNYP94C                          G
                                        C          G·GAUC··3'
                                                   A
                                                   C
                               GGAGAAUG            G
                                      A : U
                                      U : A
                                      G : C
                               P1P94  G     A
                                      U   G
```

Fig. 4C

```
                              G
                          A       U
                          C:G
                          U:A
                          C:G
                          U:A
                      C           C
                      A
                      A               A
                      A               A
                          G:U
                          G:C
                          G:C
                          G:C
                          C:G           UAAAU       A
5'···GAAAA·UCUGCC·UAA···A·U···GC                CAGC A
          : : : : :              : :       : : : :  |
          AGAUGG                 CG         GUCG U
          |              C           C  UAAAU      A
          |              U
          |               C      A
     U·CUCAAC             AUCAGCA
A    : : : : :            : : : : : :
A·GAGUUG··AAGGUA··UAGUC·U·A·AA····C
                                  U
     SNYP96                       G
                                  C          G·GAUC··3'
                                  A          A
                                  U          C
                                             G
                                             U
                                             G
                         GGAGAAUG            G
                                             G:C
                          P1P96     G     A
                                    U  G
```

Fig. 4D

Two-Part Fragmentation of the SUNYP910 Ribozyme

```
                              G
                          A       U
                           C:G
                           U:A
                           C:G
                           U:A
                           C       C
                           A
                           A       A
                           A       A
                           G:U
                    13-     G:C
                           G:C
                           G:C-42
            10             C:G      46UAAAU      A
             |             C:G                CAGC  A
5'  --P2-GAAAA-UCUGCC-UAA--  A:U---GC            ::::  |
             ::::::                   ::      GUCG  U
          AGAUGG                     CG      UAAAU   A
         | |         C            C   UAAAU      A
         | 33        U  73            U
         |          C  |          A
   U-CUCAAC              AUCAGCA
 A  ::::::               :::::: :
   A-GAGUUG--AAGGUA--UAGUC-U-A-P9.0--C:G-GAUC--3'
                                U:A
       103                      G:C
                            4c __C:G
                                A:U
                            5 — U:A
                                G:C          P1
                               G    A
                               U  G           UA
                                             C   C
                                             A   A
        P9.0= P910                           A:U
        N=P1RL                               U:A
                                             A:U
                                             G:C
                                          5' N   G
                                                 G
                                                 A
                                                 G
                                                 G
              Fig. 4E                            G 3'
```

RIBOZYME

PRODUCT (%)

SNYP9L
SNYP2HP5LAP9L
SNYP2P5LAP9L
SNYP5LAP9L
SNYP2HP5SAP9L
SNYP2P5SAP9L
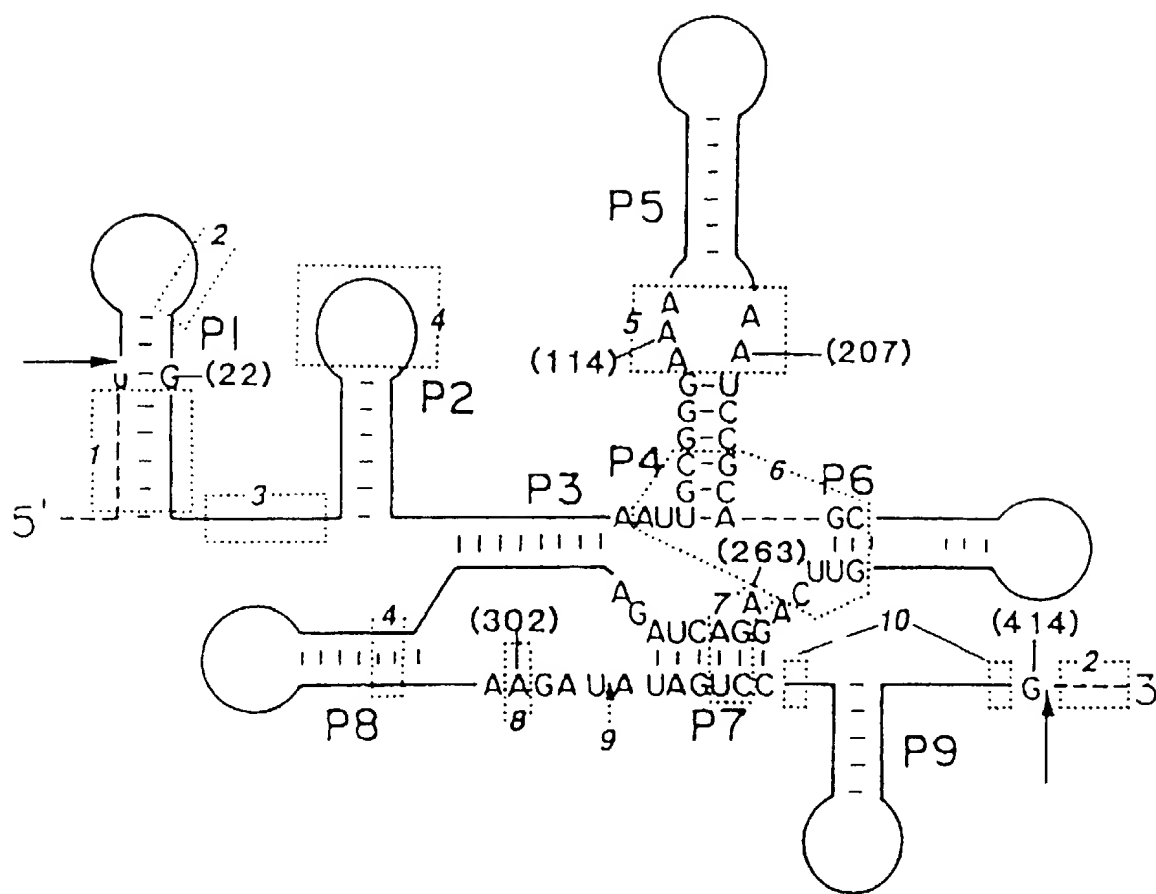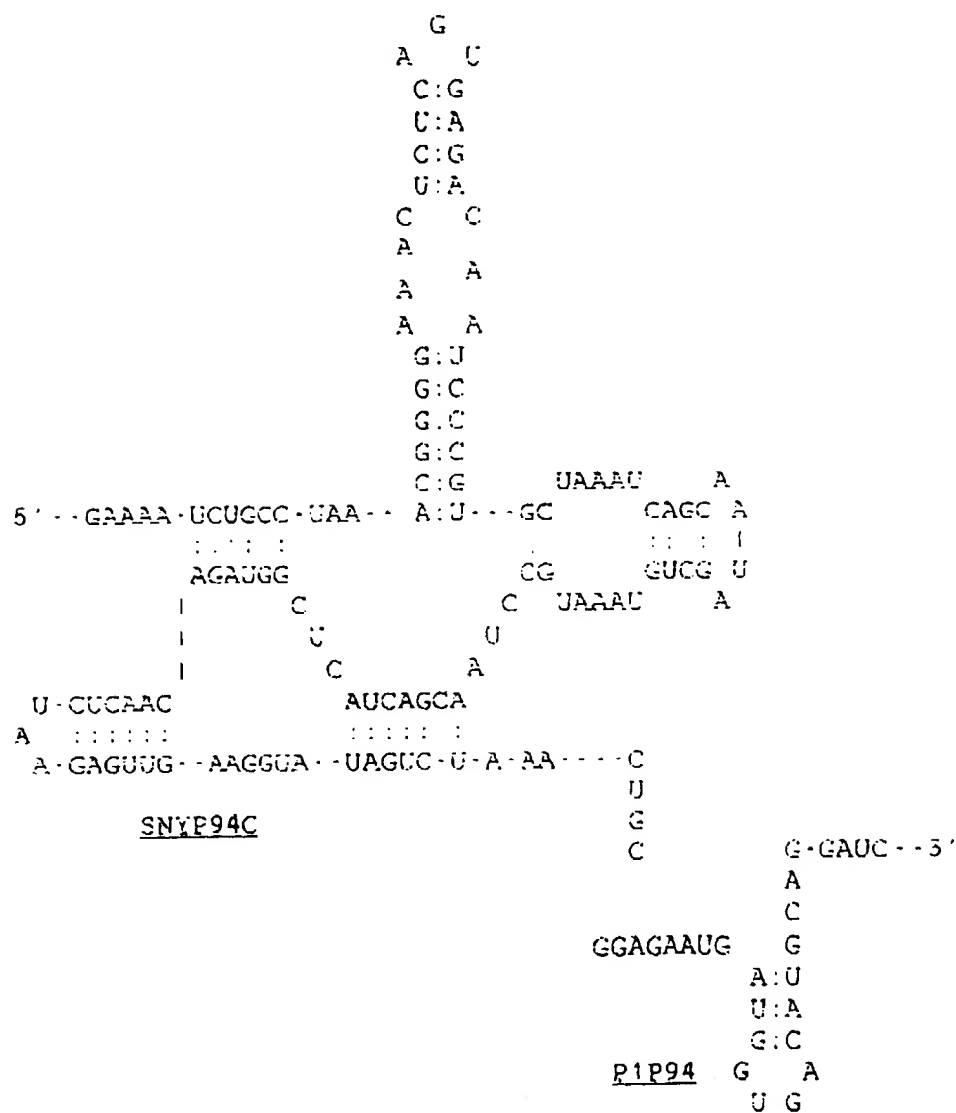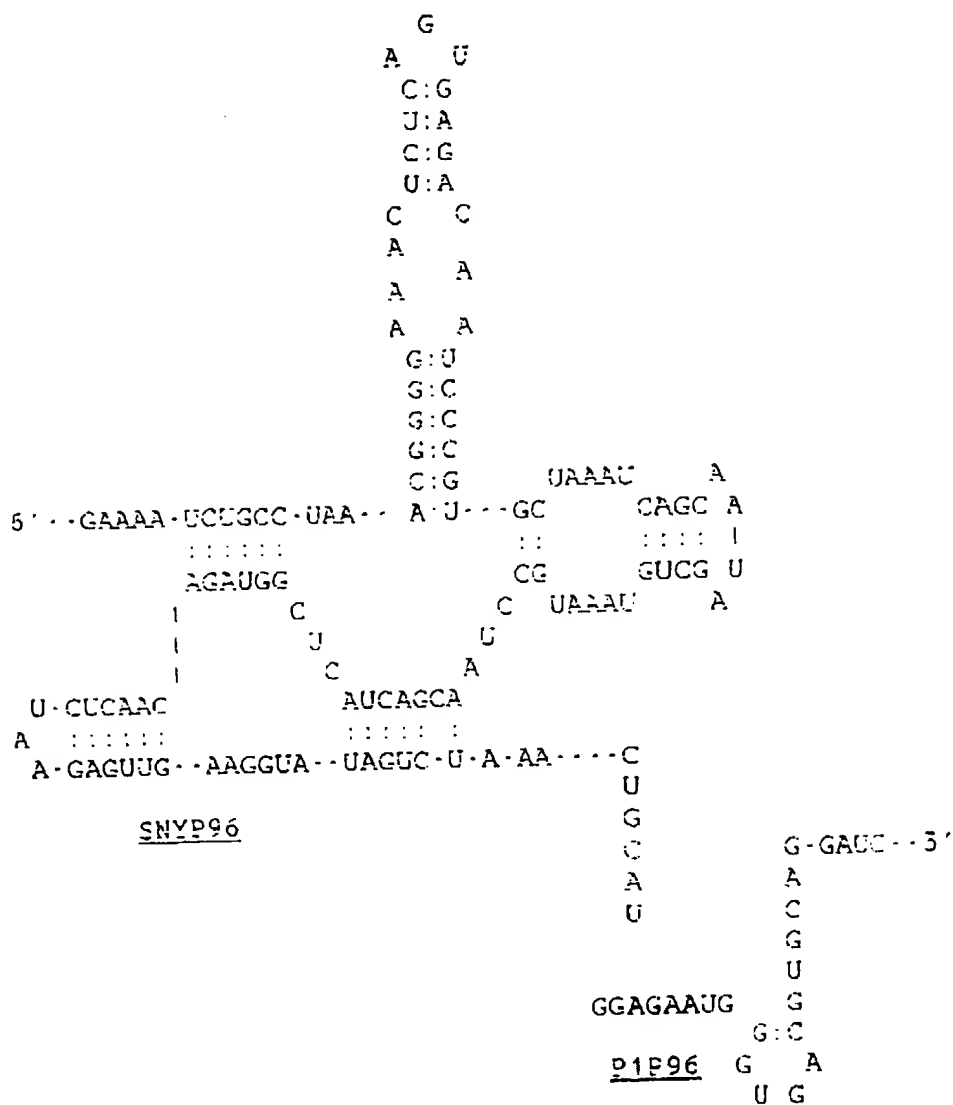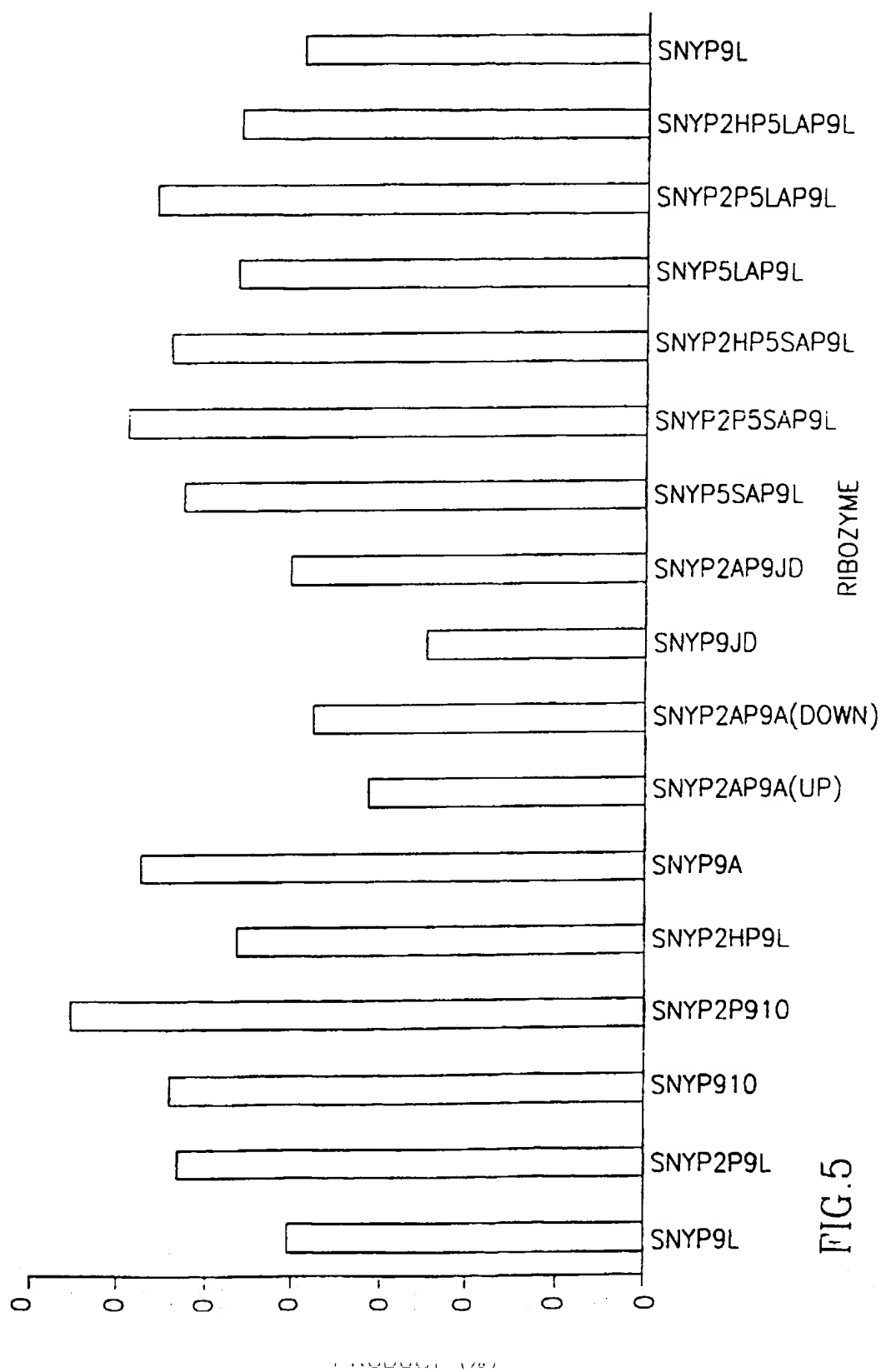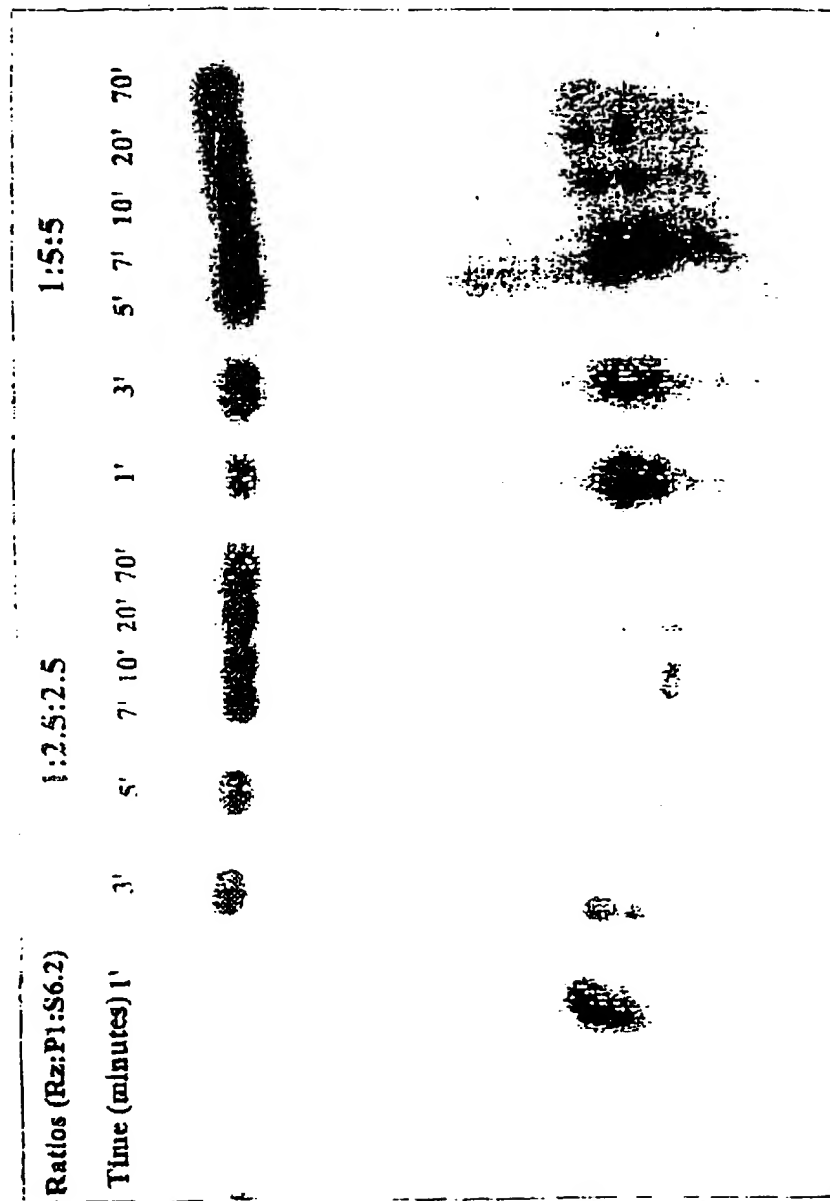SNYP5SAP9L
SNYP2AP9JD
SNYP9JD
SNYP2AP9A(DOWN)
SNYP2AP9A(UP)
SNYP9A
SNYP2HP9L
SNYP2P910
SNYP910
SNYP2P9L
SNYP9L

FIG.5

Fig. 6

Fig. 7

FIG.8

nM RATIO

◆ SERIES 1	1:2.5:2.5
■ SERIES 2	1:5:5
▲ SERIES 3	1:10:10
● SERIES 4	1:20:20

TIME.(MINUTES)

FIG.9

Fig. 10

Isopropanol[%]	EtOH[%]	Urea[M]	GITC[M]	SDS[%]
0 1 5 10 25	0 1 5 10 25	.01 .1 1 2	.01 .1 1	0 0.1 1 2.5 5

32p mode

Fig. 11

Fig. 12

GITC (M)

32p mode
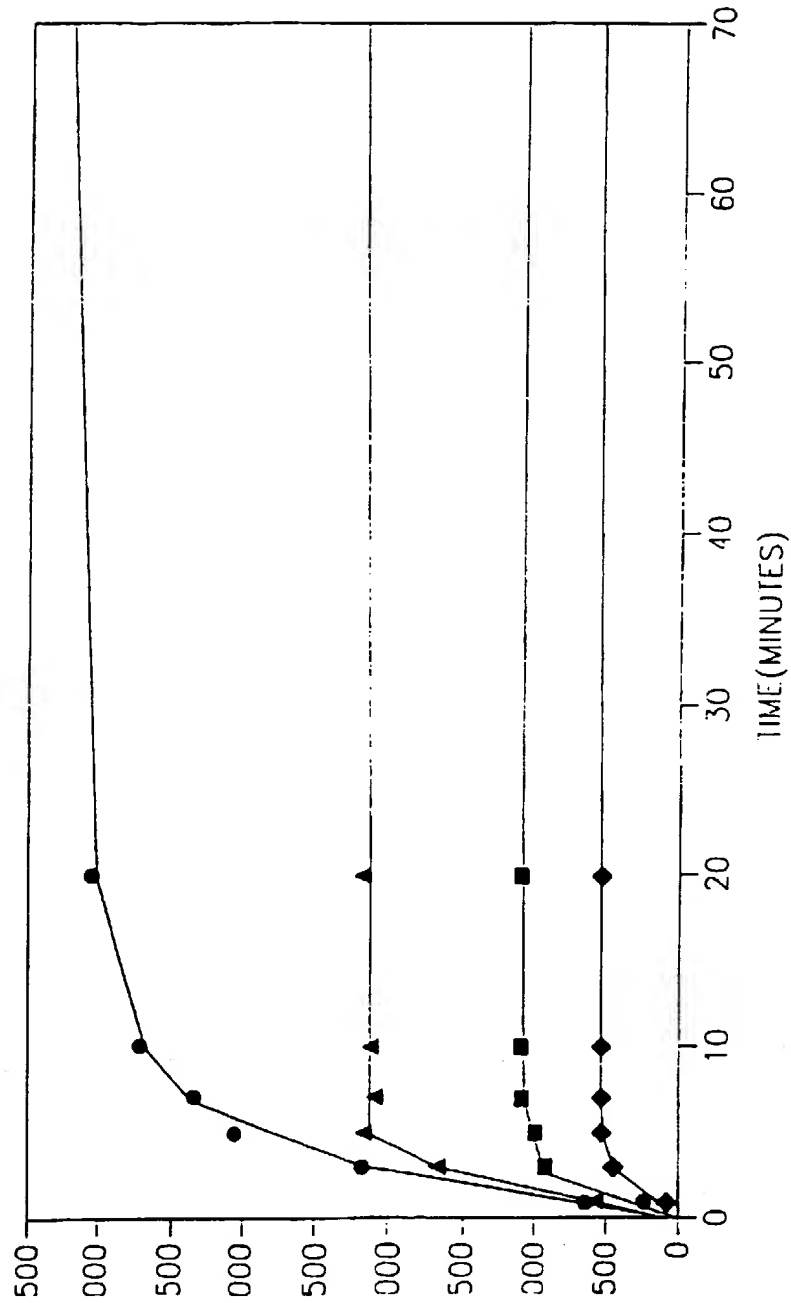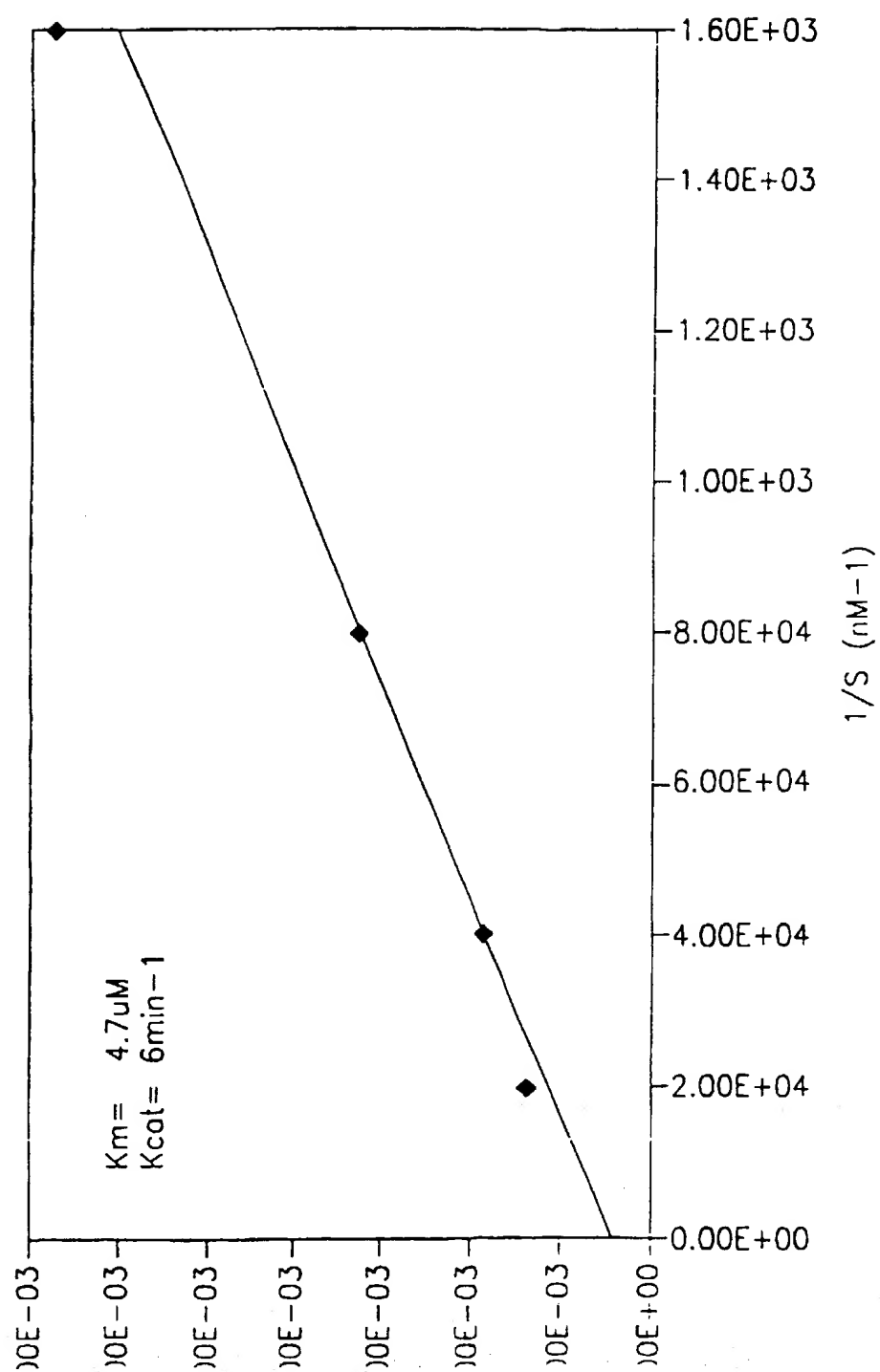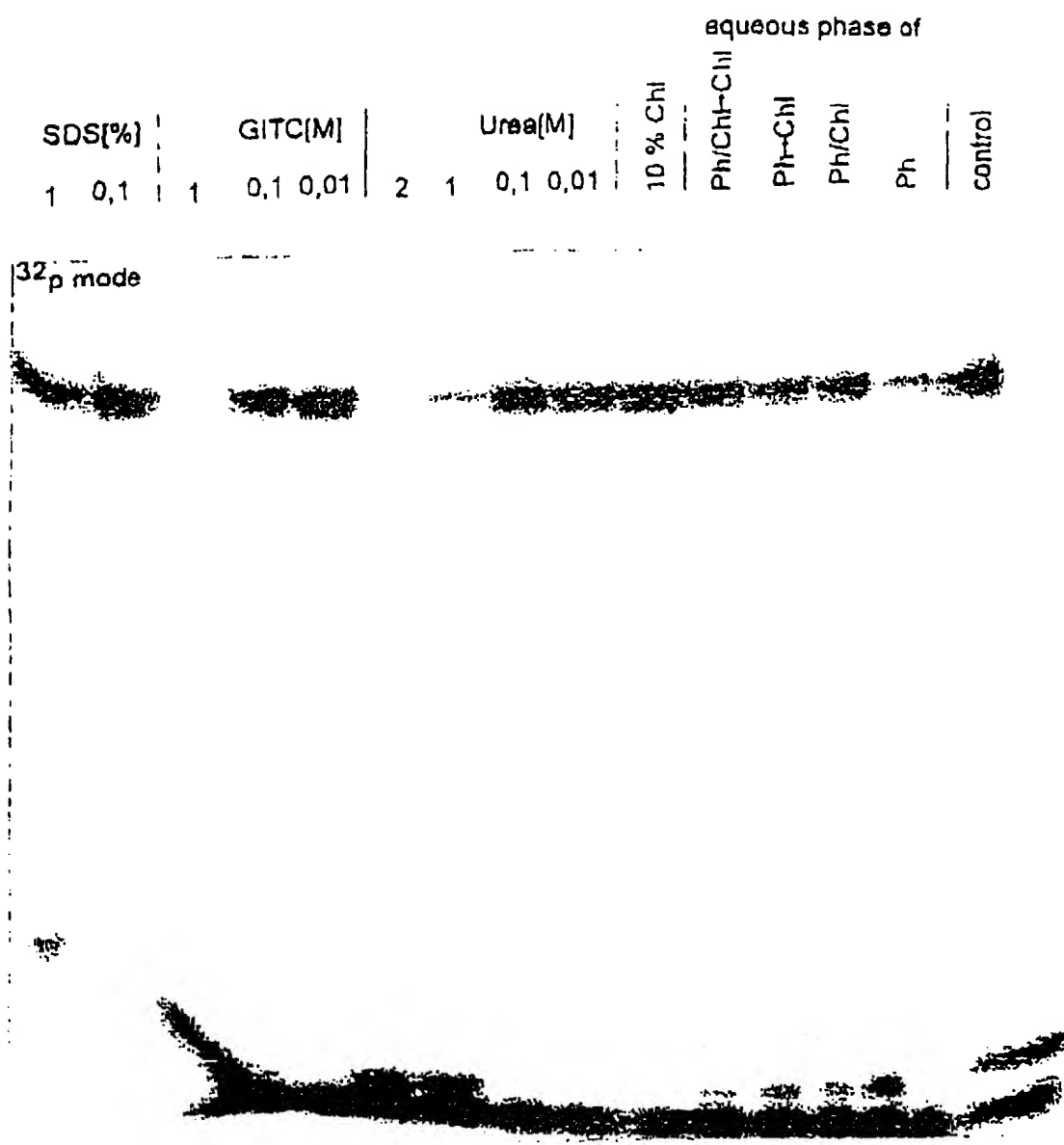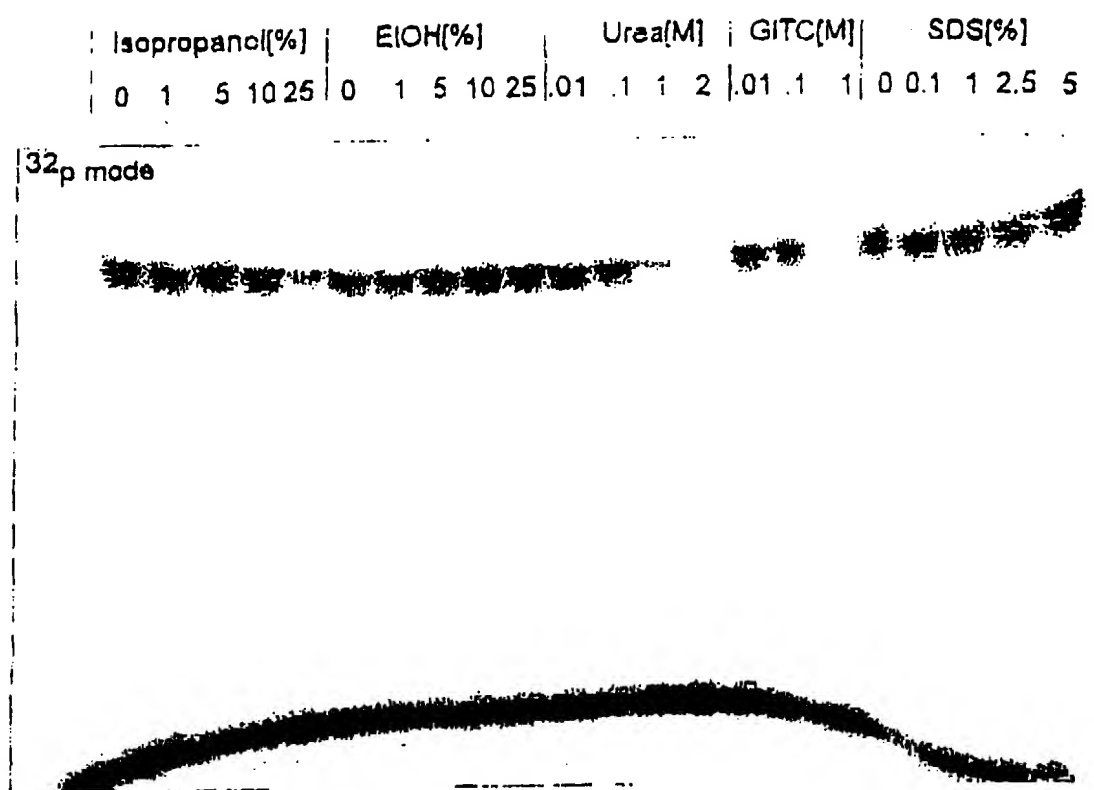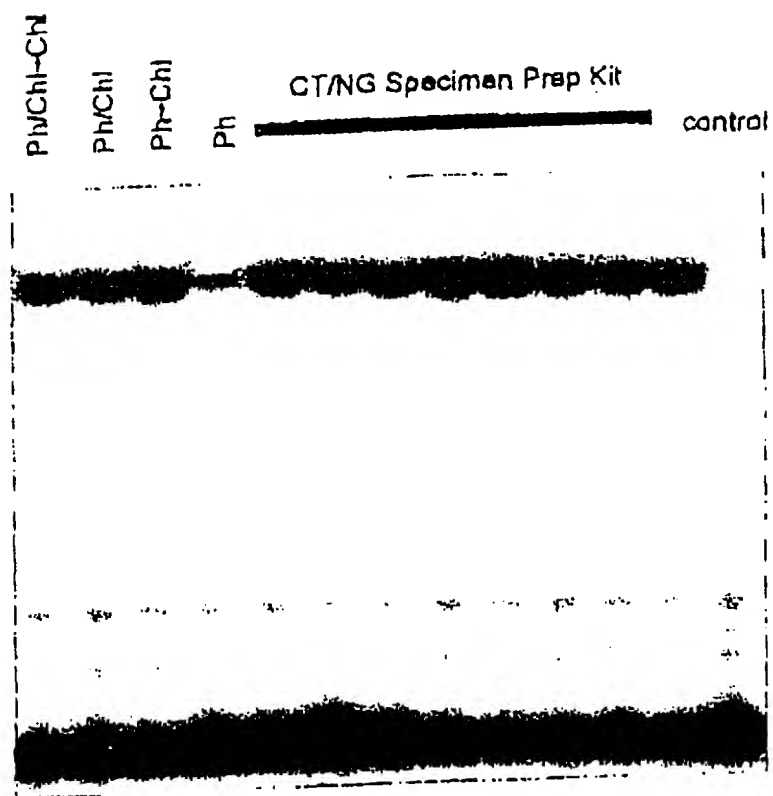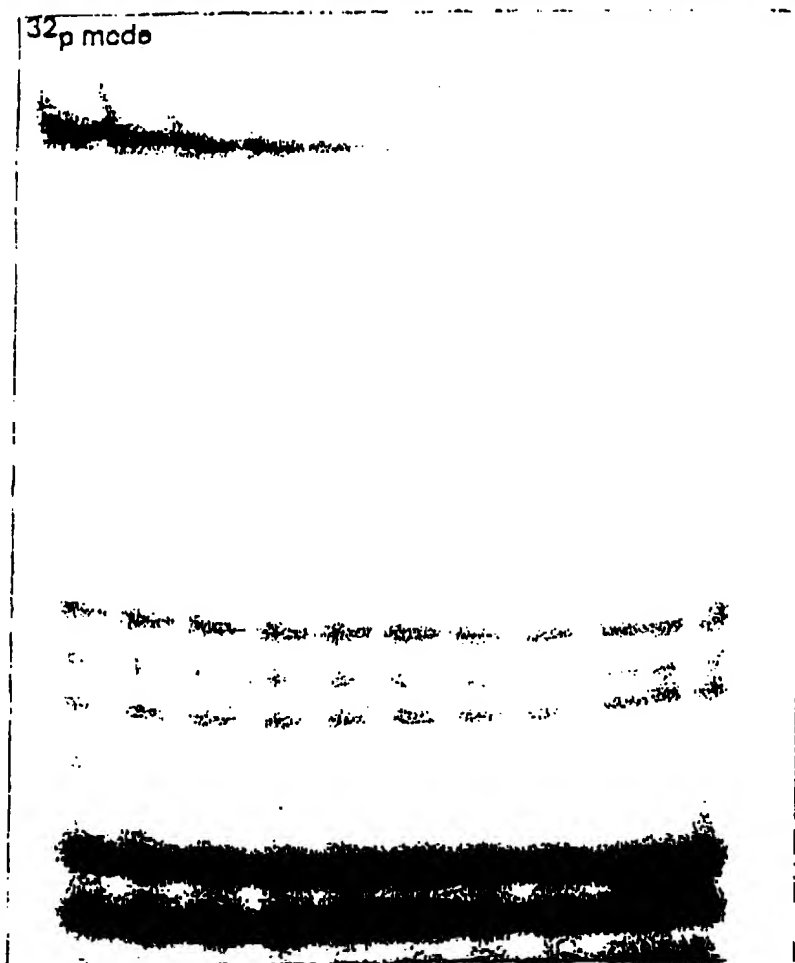
Fig. 13
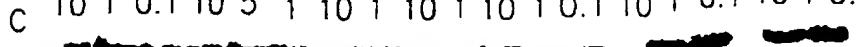
	ATA (mM)			SPERMIDINE (mM)			DMF (%)		DMSO (%)		TRITON (%)			NONIDET (%)			TWEEN-40 (%)			
C	10	1	0.1	10	5	1	10	1	10	1	10	1	0.1	10	1	0.1	10	1	0.1	
100	90		90	98		86		82		118		0		94		98				RELATIVE PRODUCT YIELD
		86	98		112	20	54		176	90	86		0		102					

FIG.14

Fig. 15

Original sunY construct:

```
                              C
                          A       U
                            C:G
                            U:A
                            C:G
                            U:A
                          C       C
                          A
                          A       A
                          A       A
                           G:U
                           G:C
                           G:C
                           G:C                              Fragment A
                           C:G        UAAAU      AAUA--3'
5'--GAAAA-UCUGCC-UAA--  A:U---GC              CAGC
        ::::::              ::           ::::
      AGAUGG                CG         GUCG--5'
      |         C            C   UAAAU
      |       A            U                   Fragment B
      |         C        A
3'--CUCAAC              AUCAGCA
      ::::::            ::::: :
5'--GAGUUG--AAGGUA--UAGUC-U-AAA--C:G-GAUC--3'
                                 U:A
                                 G:C           Fragment C
                                 C:G
                                 A:U
                                 U:A
                                 G:C
                               G     A
                               U     G
```

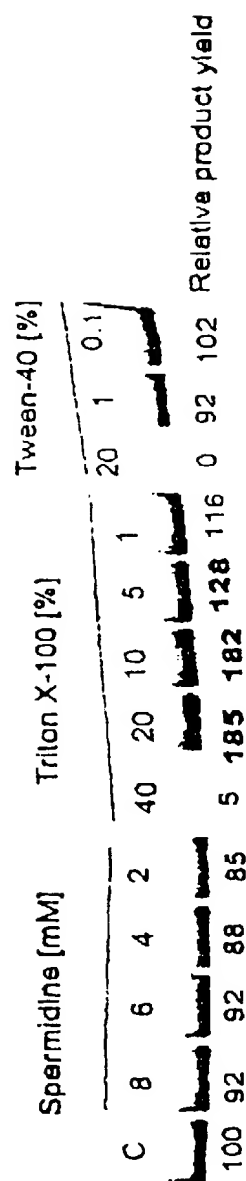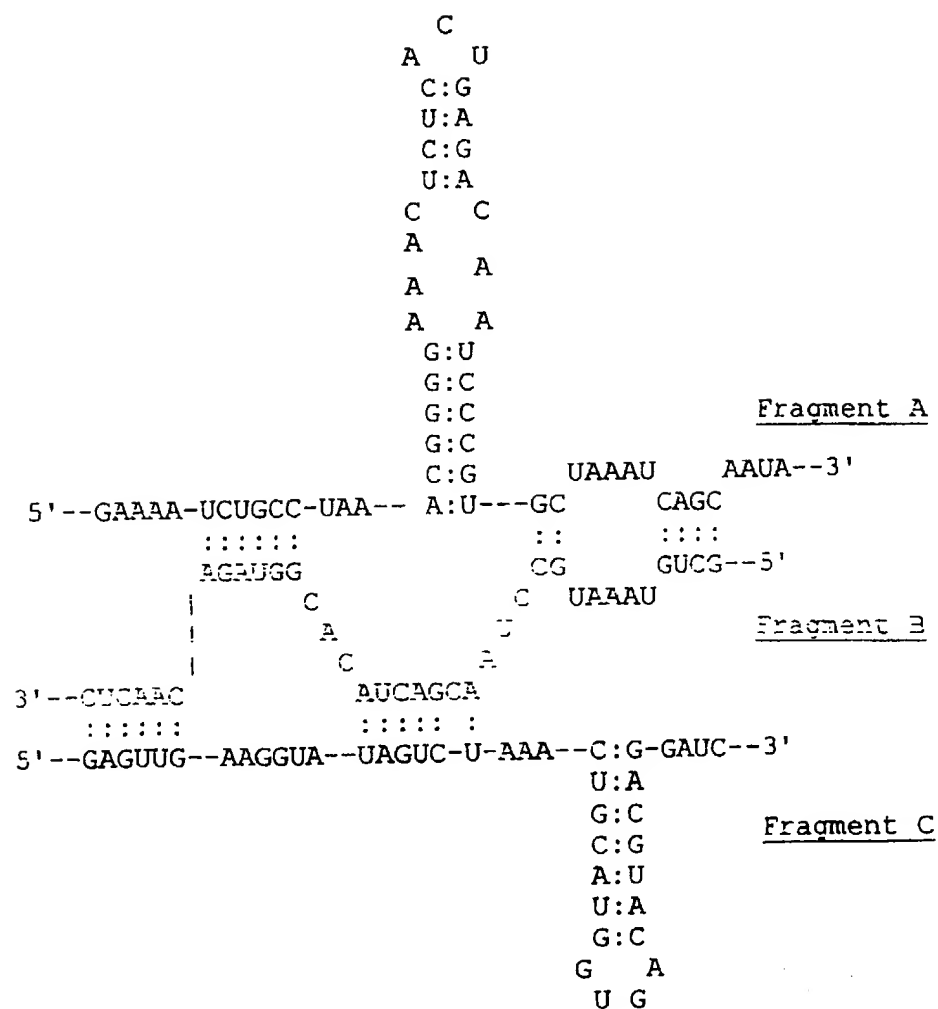**Fig. 16**

Option 29 (CT16)
Fragment B is a specific Chlamydia 16S sequence.

```
                           C
                        A     U
                        C:G
                        U:A
                        C:G
                        U:A
                        C     C
                        A
                        A     A
                        A     A
                         G:U
                         G:C
                         G:C
                         C:G
                         C:G          UUAAU      AAUA--3'
5'--GAAAA-CGCCCA-UAA--  A:U---AC          CACC
        ::::::              ::       ::::
        GCGGGU              UG       GUGG
     :                  U        U  CGAUC       --5'
     :                  A           A
     :                   U          A
3'AUGGAAU                  UUAGGCU
   ::::::                  ::::: :
5'-ACCUUUA--AAGGUA--AAUCC-A-AP9.0C:G-GAUC--3'
                            U:A
                            G:C
                            C:G
                            A:U
                            U:A
                            G:C
                          G     A
                           U  G
```
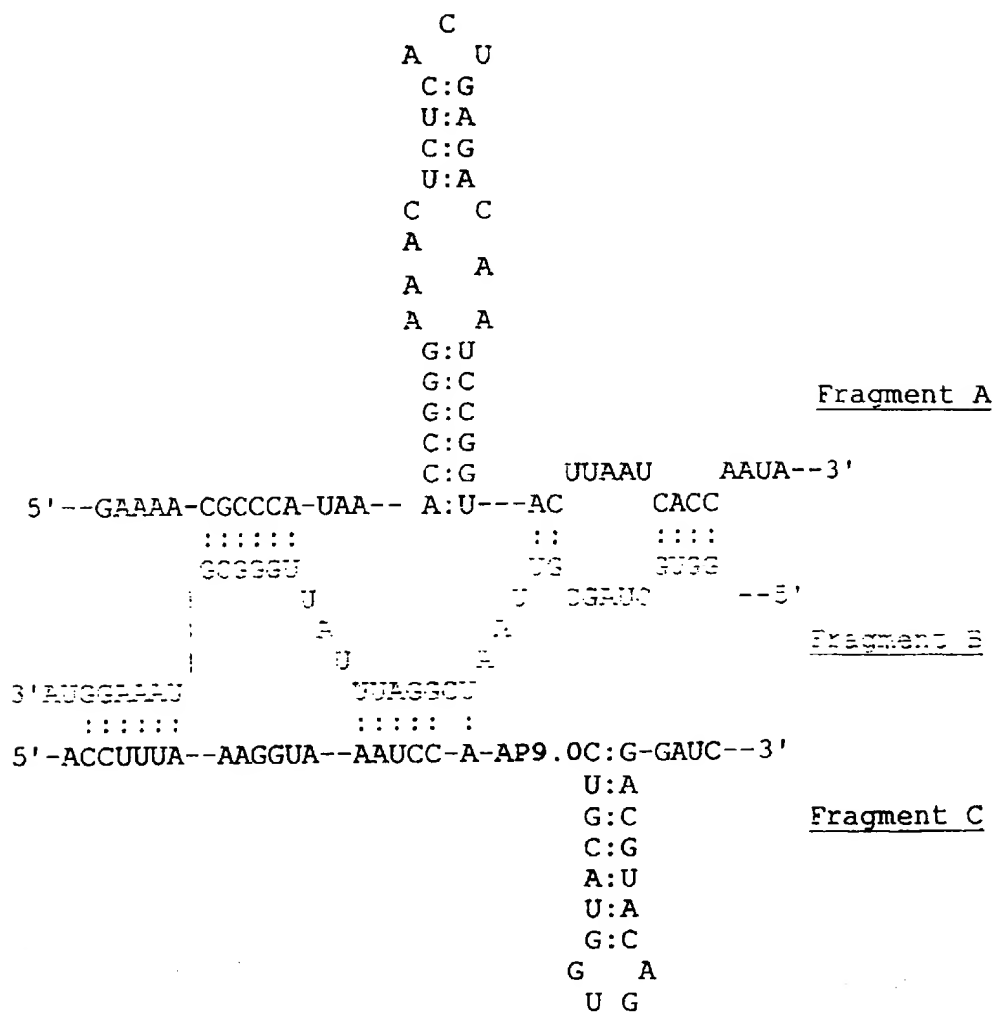
Fragment A
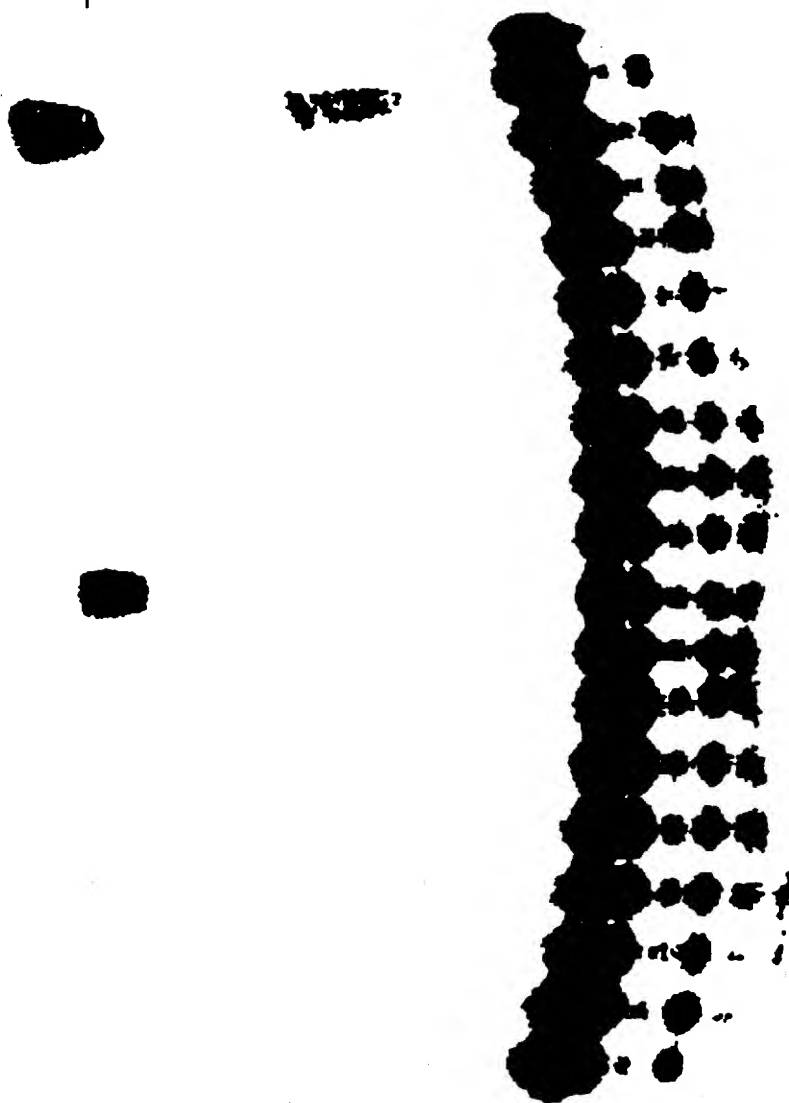
Fragment B

Fragment C

Fig. 17

21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 wt 6.2

—OPTION #

—LIGATED PRODUCT

—6.2 SUBSTRATE

FIG.18
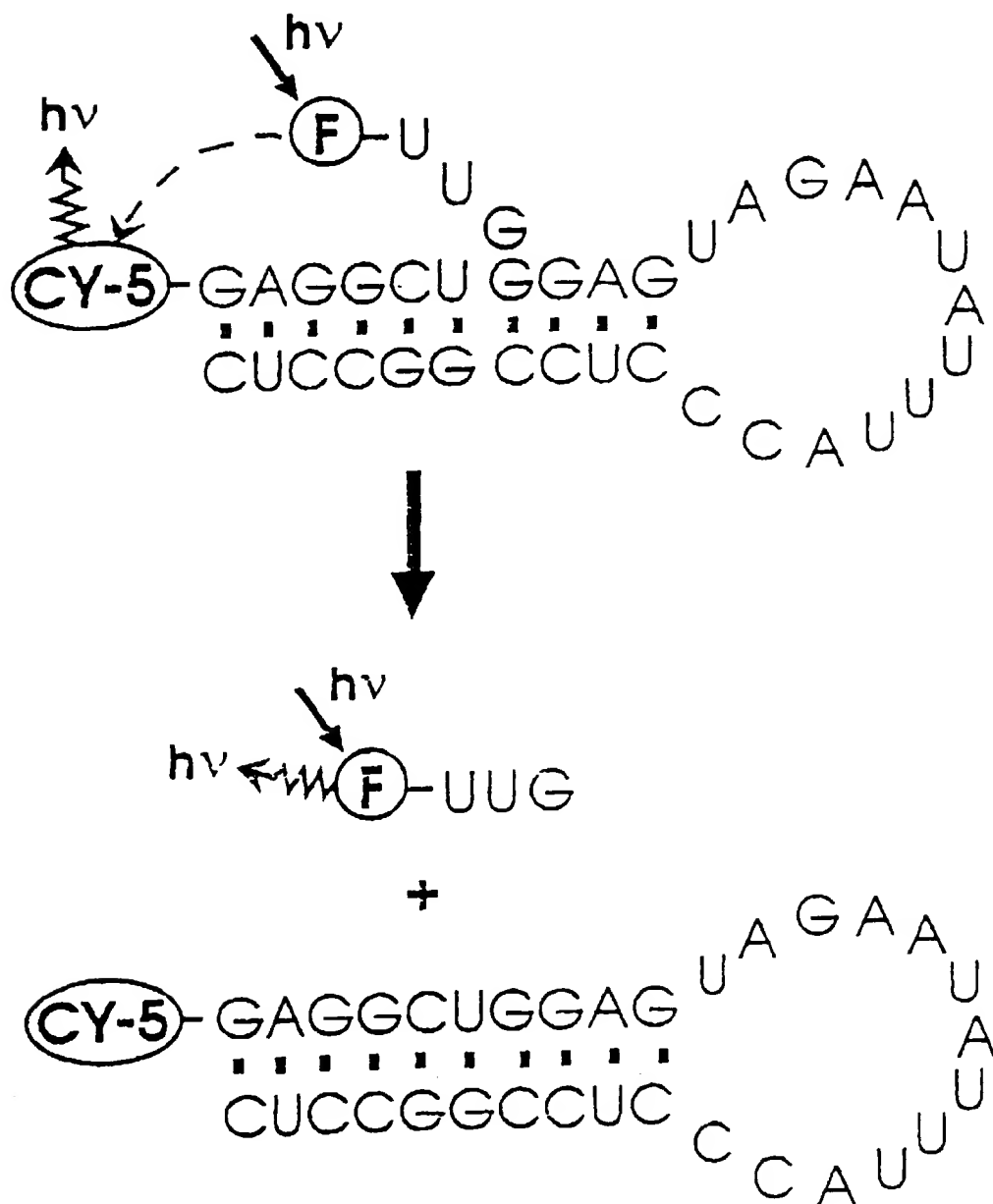
Fig.19

	NONE	100 mM GITC +BLOOD	100 mM GITC	100mM UREA	5%SDS	CONTROL
	+ −	+ −	+ −	+ −	+ −	

100   55   71   101   76

RELATIVE
PRODUCT
YIELD

FIG.20

5% SDS
100 mM UREA
NO DENATURANT
ADDED

NO RIBOZYME

100mM GITC

TIME (x 5min)

7000 6000 5000 4000 3000 2000

1 2 3 5 6 7 9 10 11 13 14 15 16 18 19 20 22 23 24 26 27 28 29 31 32 33 35 36

FIG.21

```
                  U C
              U       G
                  C-G
                  A-U
                  G-C
          PPP G-C
           HO U A
                  C G
                  A U
                  C G
                  G C
   | tag |      A UCGUG-N 90- CUCGUGAUGUCCAGUCGC 3'
                              GAGCACTACAGGTCAGCG 5'
```

Fig. 22

FIG.23